(54) Title: NOVEL CHEMILUMINESCENT FUSED POLYCYCLIC RING-CONTAINING 1,2-DIOXETANES AND ASSAYS IN WHICH THEY ARE USED

(57) Abstract

Chemiluminescent 1,2-dioxetane compounds are disclosed in which the molecule is stabilized at the 3-position on the dioxetane ring against decomposition prior to the molecule's coming in contact with a labile group-removing substance and substituted at the 4-position on the dioxetane ring with a fused polycyclic ring-containing fluorophore moiety bearing a labile ring substituent whose point of attachment to the fused polycyclic ring, in relation to this ring's point(s) of attachment to the dioxetane ring, is such that the total number of ring atoms separating these points of attachment, including the ring atoms at the points of attachment, is an odd whole number. They are useful in detecting the presence or determining the concentration of chemical or biological substances in immunoassays, chemical assays and nucleic acid probe assays.

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NOVEL CHEMILUMINESCENT FUSED POLYCYCLIC RING-CONTAINING 1,2-DIOXETANES AND ASSAYS IN WHICH THEY ARE USED

FIELD OF THE INVENTION

This invention relates to improved chemiluminescent 1,2-dioxetane compounds and novel intermediates used in their preparation. More particularly, this invention relates to

5 improved chemiluminescent 1,2-dioxetane compounds that contain labile groups removable enzymatically, chemically or electrochemically to produce fluorophores that in turn emit optically detectable energy, usually luminescence in the form of visible light, and that also contain stabilizing groups that

10 prevent these dioxetanes from decomposing before the labile group comes in contact with the substance(s) that will remove it. This invention further relates to the incorporation of such light-emitting reactants in art-recognized immunoassays, chemical assays and nucleic acid probe assays, and to their use as direct

15 chemical/physical probes, to permit an analyte, a chemical or biological substance whose presence, amount or structure is being determined, to be identified or quantified.

BACKGROUND OF THE INVENTION

Chemiluminescent 1,2-dioxetanes and their use in previously-

20 developed chemical and biochemical assays has assumed increasing importance in recent years, particularly with the advent of

enzymatically-cleavable 1,2-dioxetanes; see, for example, copending Bronstein U.S. patent application Serial No. 889,823, "Method of Detecting a Substance Using Enzymatically-Induced Decomposition of Dioxetanes", filed July 24, 1986; Bronstein et al U.S. patent application Serial No. 140,035, "Dioxetanes for Use in Assays", filed December 31, 1987; Edwards U.S. patent application Serial No. 140,197, "Synthesis of 1,2-Dioxetanes and Intermediates Therefor", filed December 31, 1987 and Voyta et al U.S. patent application Serial No. 203,263, "Chemiluminescence Enhancement", filed June 1, 1988.

It is known that chemiluminescent 1,2-dioxetanes substituted at the 4-position on the dioxetane ring with a monocyclic aromatic ring-containing fluorophore moiety can be configured to maximize their charge transfer characteristics ("emission efficiencies") in the excited state and, as a result, increase the intensity of the light emitted when such compounds decompose. This is the so-called "meta effect" discussed in Schaap et al, Tetrahedron Letters, p. 1155 (1987). The meta effect, as the name implies, is achieved by placing a labile substituent containing a bond cleavable to yield an electron-rich fluorophore moiety meta to the point of attachment of the monocyclic aromatic ring to the dioxetane ring.

Knowledge of the meta effect, however, would not have enabled those skilled in the art to predict with assurance what effect, if any, substituent placement might have on the intensity of light emitted by the decomposition of chemiluminescent 1,2-dioxetanes substituted at the 4-position on the dioxetane ring with fused polycyclic, rather than monocyclic, ring-containing

fluorophore moieties, and certainly would give no guidance whatsoever regarding the duration or the nature, i.e., the wavelength or color, of light emitted by such polycyclic moieties on decomposition.

5

SUMMARY OF THE INVENTION

This invention is concerned with chemiluminescent 1,2-dioxetanes substituted with certain fused polycyclic ring-containing fluorophore moieties. In particular, it is concerned with chemiluminescent 1,2-dioxetanes stabilized at the 3-position

10   on the dioxetane ring against decomposition prior to the molecule's coming in contact with a labile group-removing substance (e.g., an enzyme that will result in enzymatic cleavage of the dioxetane molecule) and substituted at the 4-position on the dioxetane ring with a fused polycyclic ring-

15   containing fluorophore moiety which is itself substituted in a particular fashion with a labile ring substituent containing a bond which, when cleaved, renders this fluorophore moiety electron-rich and hence renders the dioxetane decomposable to emit light.

20   It has now been discovered, quite unexpectedly, that if such fused polycyclic ring-containing fluorophore moieties are substituted in a particular pattern, i.e., if they contain at a particular ring position in relation to the polycyclic ring's point(s) of attachment to the dioxetane ring a labile ring

25   substituent having a cleavable bond they will, by virtue of this substitution pattern, decompose to emit light of unexpectedly

greater intensity in certain environments, as well as light of

longer duration, than that emitted by corresponding isomeric

compounds, i.e., otherwise identical fused polycyclic ring-

substituted 1,2-dioxetanes having the same labile ring

5   substituents on the polycyclic ring but on different ring

positions and thus in a different substitution pattern.

In contrast to other chemiluminescent materials -- for

example acridinium esters, or luminols under most conditions --

the chemiluminescent 1,2-dioxetanes of this invention, when

10   decomposed, emit light as a glow rather than a flash. The

kinetic difference between these two types of light emission is

illustrated in the following excerpt from Knox Van Dyke,

"Bioluminescence and Chemiluminescence: Instruments and

Applications" (Boca Raton, Fla.: CRC Press, 1985), Vol. 1, p. 5:
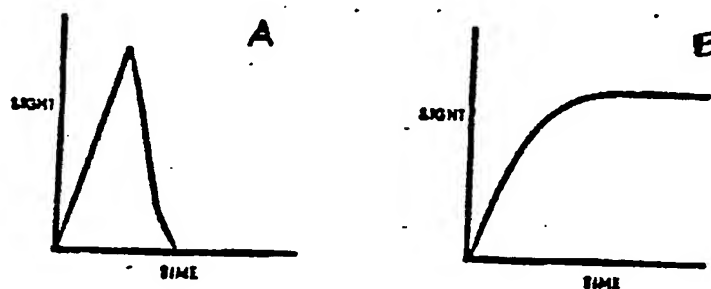
15   FIGURE 4. The production of light from chemical reaction
that produces a light peak or flash is depicted in (A) while a
steady-state production of light is depicted in (B). In general,
the measurement of light in state (B) is more convenient and
reproducible than in state (A).

20·   High intensity light emission permits the use of simple

photodetectors (silicon photodiodes or photographic film, for

example) as well as conventionally employed photomultiplier tubes

to detect the emitted light. The stability of the emission

signal produced by these enhanced intensity chemiluminescent 1,2-

dioxetanes also simplifies assay procedures in which they are

used as the means by which the analyte is identified or

5   quantified because the chemiluminescent reaction can be initiated

prior to transferring the assay mixture to the light detector.

Hence, precisely timed operations are not required.

This invention's substitution pattern is one in which the

labile ring substituent's point of attachment to the fused

10   polycyclic ring, in relation to this ring's point of attachment

to the dioxetane ring, is such that the total number of ring

atoms, e.g., ring carbon atoms, separating these two points of

attachment, including the carbon atoms at the points of

attachment, is an odd whole number. Preferably, the total number

15   of ring atoms separating these two points of attachment,

including the ring atoms at the points of attachment, will be an

odd whole number of 5 or greater. A naphthalene residue

attached by a single bond or through a spiro linkage to the

dioxetane ring at this residue's 2-carbon atom and bearing an

20   enzyme-cleavable substituent, e.g., a phosphate ester group, at

its 5- or 7-carbon atom, or a naphthalene residue attached at its

1-carbon atom and substituted at its 8-carbon atom, are typical

examples from among this invention's contemplated odd pattern

fused polycyclic aromatic hydrocarbon ring-containing fluorophore

25   moieties.

Most unexpectedly, the novel odd pattern substituted fused

polycyclic ring-containing 1,2-dioxetanes of this invention also

exhibit on decomposition a pronounced shift in the wavelength of

the light emitted, i.e., this light is a different color from that emitted by corresponding isomeric compounds which have an even rather than an odd numbered substitution pattern.

This invention is further concerned with the use of these improved chemiluminescent 1,2-dioxetanes in art-recognized immunoassays, chemical assays and nucleic acid probe assays to detect the presence or determine the concentration of chemical or biological substances, and as direct chemical/physical probes for studying the molecular structures or microstructures of various macromolecules: synthetic polymers, proteins, nucleic acids and the like.

Also, by using either two or more of the improved chemiluminescent 1,2-dioxetanes of this invention, each of which upon decomposition emits light of a different wavelength, or one or more of these compounds together with a different chemiluminescent compound, such as one of the enzymatically decomposable 1,2-dioxetanes disclosed in the aforementioned copending Bronstein, Bronstein et al and Edwards applications, or a chemically or electrochemically cleavable analog thereof, which emits light of yet another wavelength, each of such compounds being structured so as to be decomposable by a different means (e.g., one of a pair of such compounds can contain a phosphate ester group cleavable by a phosphatase and the other an α-D- or β-D-glucoside group cleavable by β-glucosidase, or one compound can contain an enzyme-cleavable group and the other a chemically cleavable group such as a hydroxyl group, an alkanoyl or aroyl ester group, or an alkyl or aryl silyloxy group), a multi-channel assay can be designed in which different cleaving means attached

to or associated with two or more different analytes will, by cleaving different cleavable dioxetane substituents, induce the emission of light of a different wavelength for each analyte being assayed.

5　　　　It is, therefore, an object of this invention to provide improved chemiluminescent 1,2-dioxetane compounds and novel intermediates used in preparing them.

A further object of this invention is to provide chemiluminescent 1,2-dioxetanes stabilized at the 3-position on
10　　the dioxetane ring against decomposition prior to the molecule's being brought into contact with a labile group-removing substance to intentionally cleave a labile substituent's bond, and substituted at the 4-position on the dioxetane ring with a fused polycyclic ring-containing fluorophore moiety which is itself
15　　substituted with a labile ring substituent containing a bond which, when cleaved, renders this fluorophore moiety electron-rich and hence renders the dioxetane decomposable to emit light, this labile ring substituent's point of attachment to the fused polycyclic ring, in relation to this ring's point of attachment
20　　to the dioxetane ring, being such that the total number of ring atoms separating these two points of attachment, including the ring atoms at the points of attachment, is an odd whole number.

Another object of this invention is to provide improved chemiluminescent 1,2-dioxetane compounds which, upon
25　　decomposition, emit light of greater intensity than the light emitted by isomers thereof and which also exhibit a shift in the wavelength of the light emitted as compared to the wavelengths

of light emitted by the corresponding even pattern substituted compounds.

A still further object of this invention is the use of these improved chemiluminescent 1,2-dioxetane compounds to generate
5   light in aqueous and non-aqueous media.

Yet another object of this invention is the use of these improved chemiluminescent 1,2-dioxetane compounds in art-recognized immunoassays, chemical assays and nucleic acid probe assays, including multi-channel assays, to detect the presence or
10   determine the concentration of chemical or biological substances, and as direct chemical/physical probes for studying the molecular structures or microstructures of various macromolecules: synthetic polymers, proteins, nucleic acids and the like.

These and other objects, as well as the nature, scope and
15   utilization of this invention, will become readily apparent to those skilled in the art from the following description, the drawings and the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the hyperchromic effect and bathochromically
20   shifted emission by 3-(2'-spiroadamantane)-4-methoxy-4-(7"-acetoxy)naphth-2'-yl-1,2-dioxetane, prepared as described in Example I infra, when decomposed with carboxylesterase as described in Example II, infra.

FIG. 2 compares the intensity and wavelength of the light
25   emitted by 3-(2'-spiroadamantane)-4-methoxy-4-(7"-acetoxy)naphth-2'-yl-1,2-dioxetane("A") with that of its 2,6-naphthalene

dioxetane acetate isomer when each of these isomers is decomposed with aqueous sodium hydroxide as described in Example III, _infra_.

FIG. 3 compares the intensity and wavelength of the light emitted by 3-(2'spiroadamantane)-4-methoxy-4-(7"-acetoxy)naphth-2'-yl-1,2-dioxetane ("A") with that of 3-(2'-spiroadamantane)-4-methoxy-4-(3"-acetoxy)phenyl -1,2-dioxetane when each is decomposed with aqueous sodium hydroxide solution as also described in Example III, _infra_.

FIG. 4 compares the intensity and wavelength of the light emitted by the 2,7-naphthalene dioxetane acetate of Example I ("A") with that of its 2,6-isomer when each of these isomers is decomposed with sodium hydroxide in aqueous methyl cyanide as described in Example IV, _infra_.

FIG. 5 compares the intensity and wavelength of the light emitted by the 2,7-naphthalene dioxetane acetate of Example I with that of its 2,6-isomer when each of these compounds is decomposed with sodium hydroxide in aqueous acetonitrile as described in Example V _infra_. In FIG. 5 "A" is the plot for the 2,7-isomer.

FIG. 6 compares the intensity and wavelength of the light emitted by the 2,7-naphthalene dioxetane acetate of Example I ("A") with that of 3-(2'-spiroadamantane)-4-methoxy-4-(3"-acetoxy)phenyl-1,2-dioxetane when each is decomposed in sodium methoxide as described in Example VI, _infra_.

FIGS. 7 and 8 show the duration of the light emitted when 3-(2'-spiroadamantane)-4-methoxy-4-(6"-acetoxy)naphth-2'-yl-1,2-dioxetane (FIG. 7) and its 2,7-isomer of this invention (FIG. 8)

are decomposed with sodium hydroxide in aqueous CH$_3$CN as described in Example VII, _infra_.

DETAILED DESCRIPTION OF THE INVENTION

5   The improved chemiluminescent 1,2-dioxetanes of this invention can be represented by the general formula:
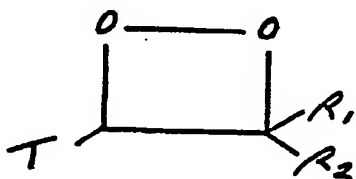
(I)

In this formula the symbol R$_1$ represents hydrogen, or a bond when R$_2$ represents a substituent bound to the dioxetane ring through a spiro linkage, or an organic substituent that does not

10   interfere with the production of light and that satisfies the valence of the dioxetane ring carbon atom to which it is attached to result in a tetravalent dioxetane ring carbon atom, such as an alkyl, aryl, aralkyl, alkaryl, heteroalkyl, heteroaryl, cycloalkyl or cycloheteroalkyl group, e.g., a straight or

15   branched chain alkyl group having from 1 to 7 carbon atoms, inclusive; a straight or branched chain hydroxyalkyl group having from 1 to 7 carbon atoms, inclusive, an -OR group in which R is a C$_1$-C$_{20}$ unbranched or branched, unsubstituted or substituted, saturated or unsaturated alkyl, cycloalkyl, cycloalkenyl, aryl,

20   aralkyl or aralkenyl group, or an organic silyloxy group, e.g., a

trialkylsilyloxy or alkyl arylsilyloxy group such as a t-butyldimethylsilyloxy or t-butyldiphenylsilyloxy group, any of which may additionally be fused to R_2 such that the emitting fragment contains a lactone ring, fused ring cycloalkyl,

5   cycloalkenyl, aryl, aralkyl or aralkenyl group, or an N, O or S heteroatom-containing group. Preferably, R_1 is a methoxy group.

The symbol R_2 represents a fused polycyclic ring-containing fluorophore moiety having a labile ring substituent containing a bond which, when cleaved, renders the fused polycyclic moiety

10   electron-rich to in turn render the dioxetane compound decomposable to emit light. This labile ring substituent's point of attachment to the fused polycyclic ring, in relation to this ring's point(s) of attachment to the dioxetane ring (single bond attachment or a spiro linkage), is such that the total number of

15   ring atoms separating these points of attachment, including the ring atoms at the points of attachment, is an odd whole number.

Included among the fused polycyclic ring compounds whose residues can be used to form this fluorophore moiety are fused polycyclic aromatic hydrocarbon ring fluorophoric compounds

20   containing from 9 to about 30 ring carbon atoms, inclusive, such as naphthalene:
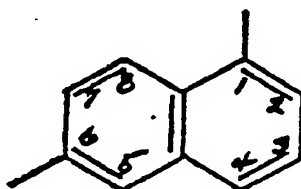
the substituent bonds indicating a 1,6-substitution pattern as in 3-(2'-spiroadamantane)-4-methoxy-4-(6"-disodiumphosphoryloxy)- naphth-1'-yl-1,2-dioxetane, pentalene, azulene, heptalene, as- indacene, s-indacene, biphenylene, perylene, acenaphthylene,

5   phenanthrene, anthracene, acephenanthrylene, aceanthrylene, triphenylene, pyrene, chrysene, naphthacene, and the like, as well as derivatives thereof substituted with one or more non- labile substituents such as a branched or straight chain alkyl group having 1 to 20 carbon atoms, inclusive, e.g., methyl, n-

10 butyl or decyl, a branched or straight chain heteroalkyl group having 1 to 7 carbon atoms, inclusive, e.g., methoxy, hydroxyethyl or hydroxypropyl; an aryl group having 1 or 2 rings, e.g., phenyl; a heteroaryl group having 1 or 2 rings, e.g., pyrrolyl or pyrazolyl; a cycloalkyl group having 3 to 7 carbon

15 atoms, inclusive, in the ring, e.g., cyclohexyl; a hetero- cycloalkyl group having 3 to 6 carbon atoms, inclusive, in the ring, e.g., dioxane; an aralkyl group having 1 or 2 rings, e.g., benzyl; an alkaryl group having 1 or 2 rings, e.g., tolyl; an electron-withdrawing group, such as a perfluoroalkyl group having

20 between 1 and 7 carbon atoms, inclusive, e.g., trifluoromethyl; a halogen; CO_2H, ZCO_2H, SO_3H, NO_2, ZNO_2, $C\equiv N$, or $ZC\equiv N$, where Z is a branched or straight chain alkyl group having 1 to 7 carbon atoms, inclusive, e.g., methyl, or an aryl group having 1 or 2 rings, e.g., phenyl; an electron-donating group, e.g., a branched

25 or straight chain C_1-C_7 alkoxy group, e.g., methoxy or ethoxy; an aralkoxy group having 1 or 2 rings, e.g., phenoxy; a branched or straight chain C_1-C_7 alkoxy group, e.g., methoxy or ethoxy; an

aralkoxy group having 1 or 2 rings, e.g., phenoxy; a branched or straight chain C_1-C_7 hydroxyalkyl group, e.g., hydroxymethyl or hydroxyethyl; a hydroxyaryl group having 1 or 2 rings, e.g., hydroxyphenyl; a branched or straight chain C_1-C_7 alkyl ester group, e.g., acetate; an aryl ester group having 1 or 2 rings, e.g., benzoate; or a heteroaryl group having 1 or 2 rings, e.g., benzoxazole, benzthiazole, benzimidazole or benztriazole.

The fused polycyclic ring portion of the fluorophore moiety represented by R_2 can also be the residue of a nonaromatic, i.e., less than fully aromatic, fused polycyclic hydrocarbon ring fluorophoric compound, having a labile ring substituent containing a bond which, when cleaved, renders the fused, less than fully aromatic polycyclic moiety electron-rich to in turn render the dioxetane compound decomposable to emit light, unsubstituted or substituted with one or more of the aforementioned non-labile substituents, and containing from 10 to about 30 ring carbon atoms, inclusive, such as fluorene, dibenzosuberene, 9,10-dihydrophenanthrene, indene, indeno [1,2-a] indene, phenalene, fluoroanthrene, and the like.

Further, the fused polycyclic ring portion of the fluorophore moiety represented by R_2 can also be the residue of a fused polycyclic aromatic or nonaromatic heterocyclic ring fluorophoric compound, e.g., benzo[b]thiophene, naphtho[2,3-b] thiophene, thianthrene, benzofuran, isobenzofuran, chromene, xanthene, phenoxathiin, quinoline, isoquinoline, phenanthridine, phenazine, phenoxazine, phenothiazine, phenanthroline, purine, 4H-quinolizine, phthalazine, naphthyridine, indole, indolizine, chroman, isochroman, indoline, isoindoline, and the like,
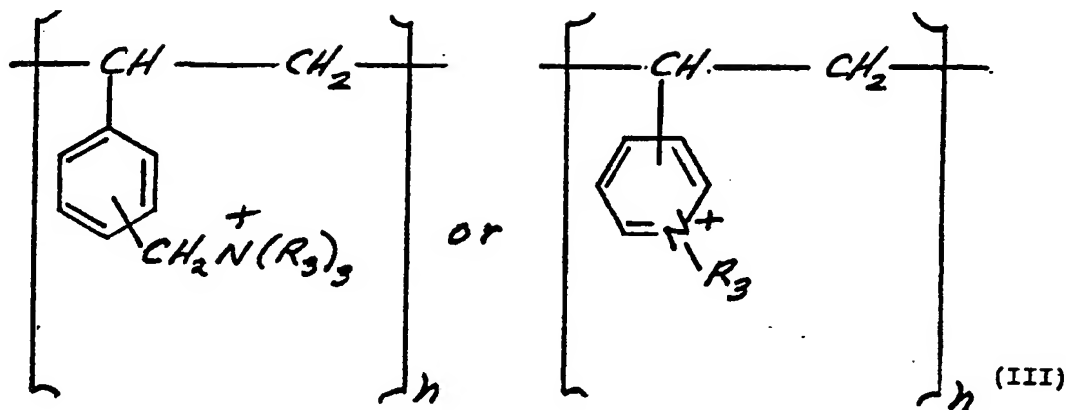
unsubstituted or substituted with one or more of the aforementioned non-labile substituents, and containing from 9 to about 30 ring atoms, inclusive, the bulk of which are carbon atoms.

5   Included among the labile ring substituents which can be positioned on a fused polycyclic ring to make up the fluorophore moieties of this invention are substituents which, when cleaved, yield an oxygen anion, a sulfur anion, or a nitrogen anion such as a sulfonamido anion. Such substituents can be chemically

10   cleavable: a hydroxyl group, an alkanoyl or aroyl ester group, or an alkyl or aryl silyloxy group, but preferably are enzymatically cleavable. Enzymatically cleavable substituents include phosphate ester groups represented by the general formula:

15

$$-O-\overset{\displaystyle O}{\overset{\displaystyle \|}{P}}-O^-\ M^+ \qquad\qquad (II)$$
$$\underset{\displaystyle O^-\ M^+}{|}$$

20   wherein M+ represents a cation such as alkali metal, e.g., sodium or potassium, ammonium, or a C_1-C_7 alkyl, aralkyl or aromatic quaternary ammonium cation, $N(R_3)_4^+$ in which each R_3 can be alkyl, e.g., methyl or ethyl, aralkyl, e.g., benzyl, or form part of a heterocyclic ring system, e.g., pyridinium, and particularly

25   the disodium salt. Such quaternary ammonium cations can also be connected through one of their quaternizing groups to a polymeric backbone, viz.

$$\left[CH - CH_2\right]_n \quad \text{(benzyl quaternary ammonium with } CH_2\overset{+}{N}(R_3)_3\text{)} \quad or \quad \left[CH - CH_2\right]_n \quad \text{(pyridinium with } \overset{+}{N}-R_3\text{)} \quad \text{(III)}$$

where n is greater than 1, or can be part of a polyquaternary ammonium salt, i.e., an ionene polymer.

Enzymatically cleavable substituents also include enzyme-cleavable alkanoyloxy groups, e.g., an acetate ester group, or an enzyme-cleavable oxacarboxylate group, 1-phospho-2,3-diacylglyceride group, 1-thio-D-glucoside group, adenosine triphosphate analog group, adenosine diphosphate analog group, adenosine monophosphate analog group, adenosine analog group, α-D-galactoside group, β-D-galactoside group, α-D-glucoside group, β-D-glucoside group, α-D-mannoside group, β-d-mannoside group, β-D-fructofuranoside group, β-D-glucosiduronate group, p-toluenesulfonyl-L-arginine ester group or p-toluenesulfonyl-L-arginine amide group.

An illustrative but by no means exhaustive listing of labile group-substituted fused polycyclic ring-containing fluorophore moieties represented by R_2 includes the following, numbered here as though they were substituents at the 4-position on the dioxetane ring: (5"-disodiumphosphoryloxy)naphth-2'-yl, (7"-disodiumphosphoryloxy)naphth-2'-yl, (6"-disodiumphosphory-loxy)naphth-1'-yl, (8"-disodiumphosphoryloxy)naphth-1'-yl, (5"-

16

acetoxy)naphth-2'-yl,(7"-acetoxy)naphth-2'-yl,(6"-
acetoxy)naphth-1'-yl, (8"-acetoxy)naphth-1'-yl, (5"-β-
galactosyloxy)naphth-2'-yl, (7"-β-galactosyloxy)naphth-2'-yl,
(6"-β-galactosyloxy)naphth-1'-yl, (5"-disodiumphosphoryl-
oxy)anthracen-2'-yl, (7"-disodiumphosphoryloxy)anthracen-2'-yl,
(6"-disodiumphosphoryloxy)anthracen-1'-yl, (8"-disodiumphos-
phoryloxy)anthracen-1'-yl, (2"-disodiumphosphoryloxy)anthracen-
10'-yl, and the like.

R$_1$ and R$_2$ together can represent a labile group-
substituted fused polycyclic ring-containing fluorophore moiety
bonded to the 4-carbon atom of the dioxetane ring through a spiro
linkage, e.g., a 6-disodiumphosphoryloxy-2-oxa-1,2,3,4-
tetrahydrophenanthr-1-ylidene group.

The symbol T represents a stabilizing group that prevents
the dioxetane compound from decomposing before the bond in the
labile ring substituent on the fused polycyclic ring-containing
fluorophore moiety is intentionally cleaved, such as an
unsubstituted or substituted cycloalkyl, aryl, including fused
aryl, or heteroaryl group, e.g., an unsubstituted cycloalkyl
group having from 6 to 12 ring carbon atoms, inclusive; a
substituted cycloalkyl group having from 6 to 12 ring carbon
atoms, inclusive, and having one or more substituents which can
be an alkyl group having from 1 to 7 carbon atoms, inclusive, or a
heteroatom group which can be an alkoxy group having from 1 to 12
carbon atoms, inclusive, such as methoxy or ethoxy, a substituted
or unsubstituted aryloxy group, such as phenoxy or
carboxyphenoxy, or an alkoxyalkyloxy group, such as methoxyethoxy
or polyethyleneoxy, or a cycloalkylidene group bonded to the 3-

carbon atom of the dioxetane ring through a spiro linkage and

having from 6 to 12 carbon atoms, inclusive, or a fused

polycycloalkylidene group bonded to the 3-carbon atom of the

dioxetane ring through a spiro linkage and having two or more

5   fused rings, each having from 5 to 12 carbon atoms, inclusive,

e.g., an adamant-2-ylidene group.

One or more of the substituents R_1, R_2 and T can also

include a substituent which enhances the water solubility of the

1,2-dioxetane, such as a carboxylic acid, e.g., acetic acid,

10   sulfonic acid, e.g., methanesulfonic acid or ethanesulfonic acid,

or quaternary amino salt group, e.g., ammonium bromide.

A preferred class of odd pattern substituted fused

polycyclic ring-containing 1,2-dioxetanes coming within the scope

of this invention are 3-(2'-spiroadamantane)-4-methoxy-4-

15   (phosphoryloxy)naphth-2'-yl-1,2-dioxetane salts such as those

represented by the formulas:

(IV)
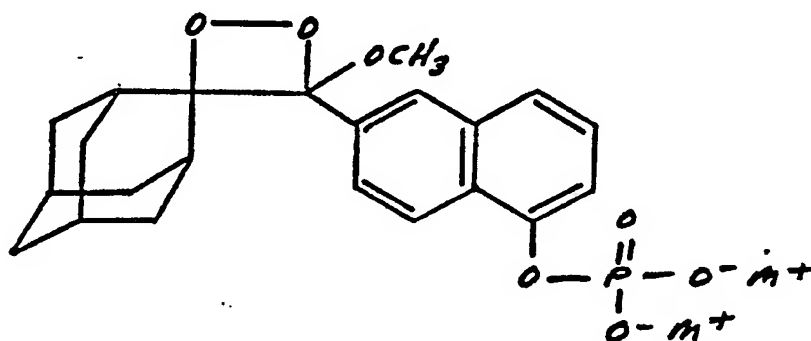
the (5"-phosphoryloxy)naphth-2'-yl isomer, and
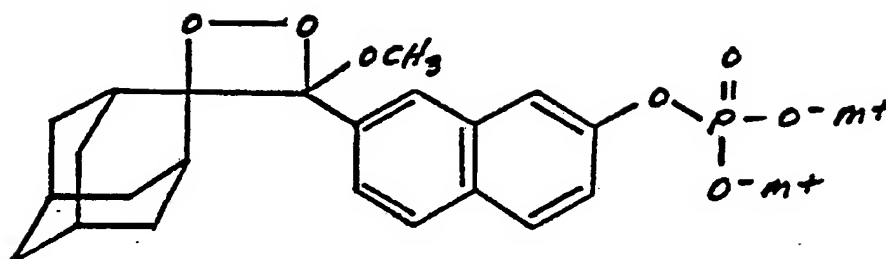
(V)

the (7"-phosphoryloxy)naphth-2'-yl isomer,

wherein M+ is as described for formula II above.

The improved chemiluminescent 1,2-dioxetanes of this

5   invention can be synthesized in known manner, e.g., by a reaction

scheme involving first the synthesis of an olefin represented by

the general formula:
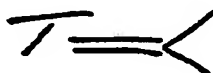
(VI)

e.g., an enol ether such as:

10
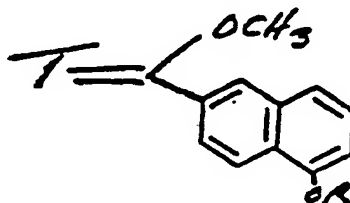
(VII)

wherein T and R are as described for formula I above, using a

Wittig reaction or the methods described in the aforementioned

copending Edwards application. Incorporation of a labile ring

15   substituent on the fused polycyclic ring and photochemical or

chemical conversion to the corresponding 1,2-dioxetane can then
be accomplished as described in the aforementioned copending
Bronstein and Edwards applications.

Key intermediates in the synthesis of the improved fused
5   polycyclic aromatic hydrocarbon ring-containing chemiluminescent
1,2-dioxetanes of this invention by methods such as those
described in the aforementioned copending Edwards application
are, first of all, fused polycyclic aromatic hydrocarbon ring-
containing fluorophoric compounds having from 9 to about 30
10   carbon atoms, inclusive, and having at least an ether
substituent, which can be an -OR group as defined above, e.g., an
alkoxy or silyloxy group such as a methoxy or t-
butyldimethylsilyloxy group, and a halo substituent, i.e., a
chlorine, bromine or iodine atom, with the total number of ring
15   carbon atoms separating these two substituents, including the
carbon atoms to which they are attached, being an odd whole
number. Such intermediates include, for example, fused
polycyclic aromatic hydrocarbon ring-containing fluorophoric
compounds such as:

20   2-chloro-7-methoxynaphthalene,

                2-bromo-7-methoxynaphthalene,

                2-iodo-7-methoxynaphthalene,

                1-chloro-8-methoxynaphthalene,

                1-bromo-8-methoxynaphthalene,

25   1-iodo-8-methoxynaphthalene,

                2-chloro-5-methoxynaphthalene,

                2-bromo-5-methoxynaphthalene,

                2-iodo-5-methoxynaphthalene,

1-chloro-6-methoxynaphthalene,

1-bromo-6-methoxynaphthalene,

1-iodo-6-methoxynaphthalene,

2-chloro-10-methoxyanthracene,

5   2-bromo-10-methoxyanthracene,

2-iodo-10-methoxyanthracene,

and the like.

These iodo-substituted ethers can be prepared from the
corresponding ether triazenes in the manner described in
10   Example I, _infra_, using an alkali metal halide such as potassium
iodide and an acidic resin. The other halo-substituted ethers
can be prepared from the corresponding diazonium salts using
Ca_2Cl_2/HCl or $CuBr/HBr$, or by chlorination or bromination and
subsequent oxidation of various methoxy 1-or 2-tetralones, as
15   described in Example VIII, _infra_.

Also key to such synthetic methods are the next two
intermediates in the sequence given, _inter alia_, in Example I,
infra, i.e., the alcohols and ketones having the general
formulas:

20   (VIII)   (IX)
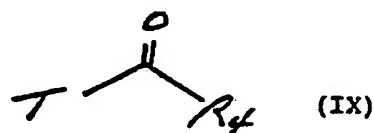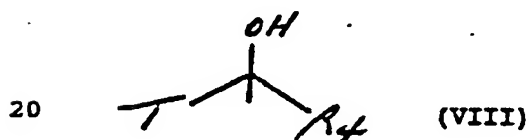
wherein T is as described for formula I above and R_4 is the ether
substituted residue of the ether and halogen substituted
fluorophoric fused polycyclic aromatic hydrocarbon ring-
containing compound, in which residue the total number of carbon
25   atoms separating the ring carbon atom to which the ether

CGK00011533

substituent is attached and the ring carbon atom through which
this residue, R_4, is attached to the remainder of the alcohol or
ketone molecule (the ring carbon atom to which the precursor
intermediate's halogen substituent was attached), including the

5   carbon atoms at the points of attachment, is, once again, an odd
whole number. Such alcohol and ketone intermediates include, for
example:

          7-methoxynaphth-2-yladamant-2'-yl alcohol,

          8-methoxynaphth-2-yladamant-1'-yl alcohol,

10   5-methoxynaphth-2-yladamant-2'-yl alcohol,

          6-methoxynaphth-2-yladamant-1'-yl alcohol,

          7-methoxynaphth-2-yladamant-2'-yl ketone,

          8-methoxynaphth-2-yladamant-1'-yl ketone,

          5-methoxynaphth-2-yl-adamant-2'-yl ketone,

15   6-methoxynaphth-2-yl-adamant-1'-yl ketone,

and the like.

     As will be apparent to those skilled in the art, the odd
patterned halo, hydroxy fused polycyclic aromatic hydrocarbons,
analogous to the above-listed haloethers and obtainable from

20   these haloethers by cleavage with HBr in acetic acid or by other
known methods, are also useful precursors of the improved
chemiluminescent 1,2-dioxetanes of this invention. For example,
the synthetic method given, _inter alia_, in Example IX _infra_ can
be modified by reacting a 6-halo-1-naphthol, e.g., 6-bromo-1-

25   naphthol, directly with two equivalents of n-butyllithium to
generate a dianion which can subsequently be reacted with one
equivalent of adamantane-2-carboxaldehyde to generate an alcohol
of general formula VIII above, where R_4 is a hydroxy rather than

an ether substituted residue. Such hydroxy-and halo-substituted
intermediates include, for example:

2-chloro-7-hydroxynaphthalene,

2-bromo-7-hydroxynaphthalene,

5   2-iodo-7-hydroxynaphthalene,

1-chloro-8-hydroxynaphthalene,

1-bromo-8-hydroxynaphthalene,

1-iodo-8-hydroxynaphthalene,

2-chloro-5-hydroxynaphthalene,

10   2-bromo-5-hydroxynaphthalene,

2-iodo-5-hydroxynaphthalene,

1-chloro-6-hydroxynaphthalene,

1-bromo-6-hydroxynaphthalene,

1-iodo-6-hydroxynaphthalene,

15   2-chloro-10-hydroxyanthracene,

2-bromo-10-hydroxyanthracene,

2-iodo-10-hydroxyanthracene, and the like.

The improved chemiluminescent 1,2-dioxetanes of this
invention are particularly useful as the means of identifying or
20   quantifying an analyte or several analytes using otherwise art-
recognized immunoassays, such as those employed to detect an
enzyme or a member of a specific binding pair, e.g., an antigen-
antibody pair or a nucleic acid paired with a probe capable of
binding to all or a portion of the nucleic acid. Such assays
25   include immunoassays used to detect a hormone such as β-HCG,
thyroid stimulating hormone (TSH), follicle stimulating hormone
(FSH), HLH or the like, cell surface receptor assays, and nucleic
acid probe assays used to detect viruses, e.g., HIV or HTLV III,

herpes simplex virus (HSV), human papiloma virus (HPV), and cytomegalovirus (CMV), or bacteria, e.g., E.Coli., and histocompatibility assays; for typical assay protocols see working examples X and XI, infra, as well as the aforementioned copending Bronstein and Bronstein et al applications. The improved chemiluminescent 1,2-dioxetanes of this invention can also be used in assays for a chemical analyte, such as potassium or sodium ions, or in assays for substances such as cholesterol or glucose in which the analyte is caused to decompose, for example using an enzyme such as cholesterol oxidase or glucose oxidase, to form a substance, e.g., hydrogen peroxide, capable in combination with another reagent of causing the chemiluminescent compound to decompose.

As noted above, by using two or more of the improved chemiluminescent 1,2-dioxetanes of this invention that each emit light of a different wavelength from the others, or by using one or more of these different colored light-emitting improved chemiluminescent 1,2-dioxetanes with one or more other chemiluminescent compounds which emit light of yet other wavelengths, each of such compounds being structured so as to be decomposable by a different means, a multi-channel assay can be designed in which different cleaving means, and especially two or more different enzymes, attached to or associated with two or more different analytes will, by cleaving different cleavable dioxetane substituents, induce the emission of light of a different wavelength for each analyte being assayed.

3-(2'-Spiroadamantane)-4-methoxy-4-(3'-disodiumphosphoryl-oxy)phenyl-1,2-dioxetane, for example, when cleaved with an

alkaline phosphatase, will emit blue light, 3-(2'-
spiroadamantane)-4-methoxy-4-(7"-acetoxy)naphth-2'-yl-1,2-
dioxetane, when cleaved with base or a carboxylesterase, will
emit green light, and 3-(2'-spiroadamantane)-4-methoxy-4-(5"-β-

5   galactosyloxy)naphth-2'-yl-1,2-dioxetane, when cleaved with a β-
galactosidase, will emit orange light. A simultaneous assay for
HSV, CMV and HPV can hence be designed using these three
chemiluminescent substances to produce light emissions of a
different color for each of these three analytes; see Example

10   XI, infra.

Light of various colors emitted when using the improved
chemiluminescent 1,2-dioxetanes of this invention singly or in
combinations as discussed above to identify or quantify various
analytes can also be used to make a permanent record of such

15   emissions on color photographic emulsions or specially sensitized
black and white high speed films. Also, these improved
chemiluminescent 1,2-dioxetanes can be used to achieve a matched
response by detectors: charged coupled devices (CCD's) or
silicon photodiodes, for example, having maximum sensitivity for

20   a color other than blue, e.g., green or red. Thus for example,
the blue light emitted when 3-(2'-spiroadamantane)-4-methoxy-4-
(6"-disodiumphosphoryloxy)naphth-2'-yl-1,2-dioxetane is cleaved
by an alkaline phosphatase to detect β-HCG can be changed to
green light simply by replacing this phosphoryloxy naphthyl

25   dioxetane with its odd pattern substituted isomer, 3-(2'-
spiroadamantane)-4-methoxy-4-(7"-disodiumphosphoryloxy)naphth-2'-
yl-1,2-dioxetane. And by using the enhanced intensity
chemiluminescent 1,2-dioxetanes of this invention together with a

light absorbing/light shifting auxiliary fluorophore/light
enhancer substance which absorbs light of one wavelength and in
turn emits light of a different wavelength, e.g., a
phycobiliprotein (phycobiliproteins are naturally-occurring

5   substances in which a fluorophore is bonded to a protein), such
as phycocyanine or phycoallocyanine, that will absorb the green
light emitted by 3-(2'-spiroadamantane)-4-methoxy-4-(7"-
disodiumphosphoryloxy)naphth-2'-yl-1,2-dioxetane and reemit this
light as red light, matched responses by color photographic

10   emulsions that exhibit a poor response to blue light, a better
response to green light but the best response to red light can
also be achieved.

    Besides the phycobiliproteins, other auxiliary fluorophores
extraneous to the light-emitting fluorophores produced by the

15   decomposition of the improved chemiluminescent 1,2-dioxetane
compounds of this invention that will accept energy, especially
light, from these light-emitting fluorophores and in turn emit
detectable energy, again preferably light, can be used when
practicing this invention. Among such auxiliary fluorophores

20   that can be used, alone or in combination, are the following
substances whose residues can be present in known 1,2-dioxetanes
and, in certain cases, if substituted with a labile ring
substituent in the appropriate odd numbered pattern, in the
improved chemiluminescent 1,2-dioxetanes of this invention as

25   well, as fluorescent chromophore groups:
    -anthracene and anthracene derivatives, e.g., 9,10-
diphenylanthracene, 9-methylanthracene, 9-anthracene
carboxaldehyde, anthrylalcohols and 9-phenylanthracene;

-rhodamine and rhodamine derivatives, e.g., rhodols,
tetramethyl rhodamine, tetraethyl rhodamine, diphenyldimethyl
rhodamine, diphenyldiethyl rhodamine and dinaphthyl rhodamine;

-fluorescein and fluorescein derivatives, e.g., 5-
iodoacetamido fluorescein, 6-iodoacetamido fluorescein and
fluorescein-5-maleimide;

-coumarin and coumarin derivatives, e.g., 7-dialkylamino-4-
methylcoumarin, 4-bromomethyl-7-methoxycoumarin and 4-
bromomethyl-7-hydroxy coumarin;

-erythrosin and erythrosin derivatives, e.g., hydroxy
erythrosins, erythrosin-5-iodoacetamide and erythrosin-5-
maleimide;

-aciridine and aciridine derivatives, e.g., hydroxy
aciridines and 9-methyl aciridine;

-pyrene and pyrene derivatives, e.g., N-(1-pyrene)
iodoacetamide, hydroxy pyrenes and 1-pyrenemethyl iodoacetate;

-stilbene and stilbene derivatives, e.g., 6,6'-
dibromostilbene and hydroxy stilbenes;

-naphthalene and naphthalene derivatives, e.g., 5-
dimethylamino naphthalene-1-sulfonic acid and hydroxy
naphthalenes;

-nitrobenzoxadiazoles and nitrobenzoxadiazole derivatives,
e.g., hydroxy nitrobenzoxadiazoles, 4-chloro-7-nitrobenz-2-oxa-
1,3-diazole, 2-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)
methylaminoacetaldehyde and 6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl-
aminohexanoic acid;

-quinoline and quinoline derivatives, e.g., 6-
hydroxyquinoline and 6-aminoquinoline;

-acridine and acridine derivatives, e.g., N-methylacridine and N-phenylacridine;

-acidoacridine and acidoacridine derivatives, e.g., 9-methylacidoacridine and hydroxy-9-methylacidoacridine;

5   -carbazole and carbazole derivatives, e.g., N-methylcarbazole and hydroxy-N-methylcarbazole;

-fluorescent cyanines, e.g., DCM (a laser dye), hydroxy cyanines, 1,6-diphenyl-1,3,5-hexatriene, 1-(4-dimethyl aminophenyl)-6-phenylhexatriene and the corresponding 1,3-

10   butadienes;

-carbocyanines and carbocyanine derivatives, e.g., phenylcarbocyanine and hydroxy carbocyanines;

-pyridinium salts, e.g., 4-(4-dialkyldiaminostyryl)N-methyl pyridinium iodate and hydroxy-substituted pyridinium salts;

15   -oxonols; and

-resorofins and hydroxy resorofins.

When such auxiliary fluorophores are bonded to a chemiluminescent compound, they are preferably bonded to the portion of the chemiluminescent compound that, upon

20   decomposition, forms a fragment containing the fluorophore portion of the chemiluminescent compound's molecule. In this way energy transfer is enhanced due to the two fluorophores being in close proximity to one another and by beneficial spatial arrangements provided by the rigidity of the microenvironment.

25   Auxiliary fluorophores that are insoluble or partially insoluble in aqueous medium can be solubilized by first grafting them onto solubilizing molecules, e.g., water soluble oligomer or polymer molecules.

And, in all cases, enhancement of the intensity of the light emitted by decomposition of the improved chemiluminescent 1,2-dioxetane compounds of this invention in aqueous media can be achieved by the methods disclosed in the aforementioned Voyta et

5   al application.

.In order that those skilled in the art can more fully understand this invention, the following examples are set forth. These examples are given solely for purposes of illustration, and should not be considered as expressing limitations unless so set

10   forth in the appended claims. All parts and percentages are by weight, unless otherwise stated.

EXAMPLE I

[Synthesis of 3-(2'-spiroadamantane)-4-methoxy-4-(7"-acetoxy)naphth-2'-yl-1,2-dioxetane]

15   <u>2-Amino-7-hydroxynaphthalene</u>. To a solution of 2,7-dihydroxynaphthalene (27.56 g, 0.172 mol) in 30% NH_4OH (200 ml) was added $NaHSO_3$ (19.03 g, 0.183 mol) at 0°C with stirring. The Bucherer reaction was then carried out by heating the solution in a steel bomb at 150°C for 4.5 hours, giving both mono- and

20   diaminonaphthalenes in a 1:1 ratio. Subsequently, the cooled product mixture was dissolved in $EtOAc/CH_3CN$ (ethyl acetate/methyl cyanide), partitioned with 1M NaOH, followed by washing the organic layer with NaOH until the washes were clear. The combined NaOH layers were acidified to pH 1, partitioned with

25   minimal EtOAc to remove traces of starting material and then carefully neutralized to pH 7 with NaOH pellets. Upon

partitioning the aqueous layer with EtOAc, the organic layer was dried over Na_2SO_4 and evaporated to yield 12.49 g (45.7%) of 2-amino-7-hydroxynaphthalene as light tan crystals, m.p. 186-188°C.

IR (Nujol, cm^{-1}): 3401, 3323, 3313, 1629, 1512, 1301, 1216, 883, 831

2-Amino-7-methoxynaphthalene. 2-Amino-7-hydroxynaphthalene (18.6 g, 0.117 mol) was added in portions to hexane-rinsed NaH (60%, 5.1 g, 0.127 mol) in dry DMF (dimethylformamide; 100 ml) at 0°C. The solution was stirred under argon for 10 minutes until hydrogen evolution had ceased, whereupon dimethyl sulfate (11 ml, 0.116 mol) was added dropwise via syringe at 0°C until O-methylation was complete by TLC monitoring. The reaction was carefully quenched with 6N HCl to pH 1, and partitioned between ether and water, thus separating the naphthylamine hydrochloride from organic impurities. After washing the ether layer with 6N HCl, the aqueous layers were combined, neutralized with NaOH pellets to pH 8 and partitioned with EtOAc to recover the free amine in the organic layer. The basic aqueous layer was washed twice with EtOAc, and the combined EtOAc layers were dried over Na_2SO_4 and evaporated. Upon removal of DMF under high vacuum, the product mixture crystallized. Crystals of 2-amino-7-methoxynaphthalene (19.4 g, 96.5%) were collected from MeOH, m.p. 157°C.

IR (Nujol, cm^{-1}): 3403, 3318, 1628, 1511, 1213, 1028, 871, 826

NMR ($CDCl_3$, ppm): 3.84 (2H, br s); 3.90 (3H, s); 6.73-7.00 (4H, m); 7.54-7.60 (2H)

<u>2-Iodo-7-methoxynaphthalene</u>. Diazotization of 2-amino-7-
methoxynaphthalene (17.02 g, 0.098 mol) in 20% aqueous HCl (81.4
ml, 0.197 mol HCl), proceeded smoothly by adding NaNO$_2$ (7.11 g,
0.103 mol) in 65 ml H$_2$O dropwise at 0°C. The reaction was

5   stirred for 1 hour, adding a small amount of EtOAc to reduce
foaming as needed. To form the triazene, a solution of K$_2$CO$_3$
(20.41 g, 0.148 mol), diethylamine (15.3 ml, 0.148 mol) and H$_2$O
(60 ml) was added dropwise to the cooled diazonium salt. After
adding ethyl ether (30 ml) to dissolve the newly formed

10   naphthalene triazene, followed by stirring for 35 minutes at low
temperature, the reaction solution was poured into a separatory
funnel and the aqueous layer was drawn off. The ether layer was
washed with H$_2$O, dried over Na$_2$SO$_4$ and evaporated under reduced
pressure with low heat, giving an orange oil. The crude

15   diethyltriazene was used immediately without purification.

2-(3',3'-Diethyl)triazo-7-methoxynaphthalene, dissolved in
120 ml dry CH$_3$CN, was added dropwise (1 drop/4 seconds) to a
slurry of ground KI (49.12 g, 0.296 mol), Amberlyst XN-1010
acidic resin (Aldrich Chemical Co.: 37.3 g), and ground molecular

20   sieves in dry DMF/CH$_3$CN (50 ml/150 ml) at 70°C overnight. Upon
complete conversion of the triazene to the iodide, the cooled
solution was carefully decanted into a separatory funnel, rinsing
the resin with two EtOAc washes. The organic material was
partitioned between EtOAc and H$_2$O, collecting the EtOAc layer,

25   which was dried over Na$_2$SO$_4$ and evaporated.

To purify the iodonaphthalene, the crude reaction mixture
was dissolved in CH$_3$CN and partitioned with hexanes until all
desired iodide had been removed from the CH$_3$CN layer. The

hexanes solution was evaporated to yield, upon addition of petroleum ether, acrid orange crystals of 2-iodo-7-methoxynaphthalene (10.36 g, 33.1% m.p. 116°C). [Literature reference: J.Org.Chem., (1983) 48, 4396.]

IR ($CHCl_3$, cm^{-1}): 3005, 1625, 1504, 1458, 1394, 1359, 1250, 1176, 1126, 1031, 958, 916, 886, 841, 631

NMR ($CDCl_3$, ppm): 3.82 (3H, s); 6.91 (1H, d, J=2.44 Hz); 7.05 (1H, dd, J=8.9, 2.5 Hz); 7.39 (1H, d, J=8.47 Hz); 7.05 (1H, dd, J=8.9, 2.5 Hz); 7.39 (1H, d, J=8.47 Hz); 7.47 (1H, d, J=8.46 Hz); 7.59 (1H, d, J=9.0 Hz); 8.04 (1H, s)

By using halogenating agents such as Cu_2Cl_2/HCl and CuBr/HBr in combination with the diazonium salt intermediate instead of the triazene, 2-chloro-7-methoxynaphthalene and 2-bromo-7-methoxynaphthalene are obtained.

7-Methoxynaphth-2-yladamant-2'-yl alcohol. Lithiation of 2-iodo-7-methoxynaphthalene (508.1 mg, 1.79 mmol) in 5 ml anhydrous ether proceeded cleanly upon addition of n-BuLi (n-butyllithium; 1.6M, 1.2 ml) at 0°C under argon. Dropwise addition of adamantane-2-carboxaldehyde, dissolved in anhydrous ether (2 ml), to the naphthyllithium solution immediately yielded the coupled lithium alkoxide. The reaction was quenched by partitioning between EtOAc/H_2O, washing the aqueous layer twice with EtOAC to completely recover the polar alcohols. The combined organic layers were dried over Na_2SO_4, evaporated and the residual oil was chromatographed on silica gel, eluting with 20% EtOAc/hexanes, to give an off-white crystalline alcohol mixture (437.9 mg, 76%).

IR, less polar alcohol (CHCl$_3$, cm^{-1}): 3601, 3005, 2905, 2846, 1632, 1607, 1515, 1465, 1391, 1265, 1175, 1125, 1034, 895, 845, 705

IR, polar alcohol (CHCl$_3$, cm^{-1}): 3589, 3005, 2905, 2849, 1631, 1609, 1515, 1465, 1393, 1259, 1137, 1129, 1035, 845, 805, 680

7-Methoxynaphth-2-yladamant-2'-yl ketone. The 7-methoxynaphth-2-yladamant-2'-yl epimeric alcohols (238 mg, 0.739 mmol) in 6 ml acetone were oxidized by dropwise addition of Jones reagent at 0°C until analytical TLC indicated complete conversion to the ketone. The reaction mixture was partitioned between EtOAc and H$_2$O and the aqueous layer was washed three times with EtOAc. The combined EtOAc layers were washed with minimal saturated NaHCO$_3$ solution, dried through a Na$_2$SO$_4$ column, and evaporated to a light yellow oil. The crude ketone was used without purification. A small amount was chromatographed (5% EtOAc/hexanes) and crystallized from acetone as a fine white powder, m.p. 116°C.

IR (CHCl$_3$, cm^{-1}): 2908, 2846, 1674 (C=O), 1629, 1605, 1466, 1272, 1257, 1179, 1132, 1126, 1105, 1035, 844

NMR (CDCl$_3$, ppm): 1.59-2.13 (12H); 2.39 (2H, s); 3.59 (1H, s); 3.94 (3H, s); 7.23 (1H, s); 7.245 (1H, d, J=3.61 Hz); 7.74 (1H, d, J=3.17 Hz); 7.76 (1H, d, J=8.30 Hz); 7.80 (1H, d, J=8.36 Hz); 8.24 (1H, s)

Methoxy(7-methoxynaphth-2-yl)methylene adamantane. Anion formation of 7-methoxynaphth-2-yladamant-2'-yl ketone (236 mg, 0.739 mmol) occurred at 50°C in the presence of potassium t-butoxide (150.6 mg, 1.34 mmol) in 3.5 ml of DMSO under argon.

After stirring for 5 minutes, addition of dimethyl sulfate (120 μl, 1.27 mmol) gave O-methylation, with a trace of ketone remaining. The cooled solution was partitioned with EtOAc/H_2O and the EtOAc layer was dried through a Na_2SO_4 column and

5 evaporated. A small amount of the residual oil was separated on an analytical TLC plate (silica gel) for spectroscopic characterization. The crude enol ether was used in the next step without purification.

IR ($CHCl_3$, cm^{-1}): 2905, 2843, 1627, 1604, 1510, 1463, 1214,

10 1124, 1091, 1033, 848

<u>Methoxy(7-acetoxynaphth-2-yl)methylene adamantane</u>. Crude methoxy(7-methoxynaphth-2-yl)methylene adamantane (247 mg, 0.739 mmol) was added to sodium ethylthiolate (62 mg, 0.743 mmol) in dry DMF (4 ml) and the solution was refluxed under argon for 4

15 hours deprotecting the aromatic methoxy moiety to the naphthol. Excess sodium ethanethiolate (122 mg, 1.45 mmol) was added in two portions during the reflux to push the demethylation to completion. The naphthol was worked up by partitioning the cooled solution between EtOAc and minimal H_2O. Traces of

20 naphthol in the aqueous layer were recovered with two EtOAc washes. The EtOAc layers were dried and evaporated. The crude naphthol was immediately acylated upon dissolution in acetic anhydride (1 ml) and pyridine (2 ml) with gentle warming to 50°C. After evaporation, the reaction mixture was chromatographed on a

25 silica gel column prewashed with 10% EtOAc/hexanes containing 1% triethylamine, eluting with the same EtOAc/hexanes/TEA solvent mixture. The four-step reaction sequence--oxidation, enol ether formation, aromatic demethylation and acylation--gave 69.3 mg

(25.8%) of methoxy(7-acetoxynaphth-2-yl)methylene adamantane, m.p. 125°C.

IR (CH$_2$Cl$_2$, cm^{-1}): 2897; 2847, 1757, 1629, 1606, 1508, 1448, 1372, 1212, 1198, 1157, 1104, 1092, 1083, 1017, 968, 919, 849

NMR (CDCl$_3$, ppm): 1.8-2.0 (13H); 2.38 (3H, s); 2.69 (1H, br s); 3.3 (3H, s); 7.21 (1H, dd, J=9.0, 2.5 Hz); 7.44 (1H, dd, J=8.5, 1.5 Hz); 7.54 (1H, d, J=1.6 Hz); 7.71 (1H,s); 7.82 (1H, d, J=8.0 Hz); 7.84 (1H, d, J=8.9 Hz)

3-(2'-spiroadamantane)-4-methoxy-4-(7"-acetoxy)naphth-2'-yl-1,2-dioxetane. A solution of methoxy(7-acetoxynaphth-2-yl)-methylene admanantane (29.0 mg, 0.080 mmol) and methylene blue adsorbed on silica gel (97.9 mg, 2.6 mg dye/g SiO$_2$) in methylene chloride (12 ml) was irradiated with a 250 Watt high pressure sodium lamp at 10°C while passing a stream of oxygen through the solution. A 5 mil thick piece of Kapton polyimide film (DuPont) placed between the lamp and the reaction mixture filtered unwanted UV radiation. TLC and UV monitoring showed complete dioxetane formation after irradiating for 25 minutes. The sensitizer was removed by filtration, the solvent was evaporated and the resulting crude oil was chromatographed on silica gel, eluting with 10% THF (tetrahydrofuran) /hexanes to give 26.0 mg (82%) of the 7"-acetoxynaphth-2'-yl dioxetane.

Starting with 1,8-dihydroxynaphthalene and proceeding through the same synthesis as in Example I gives:

1-amino-8-hydroxynaphthalene,

1-amino-8-methoxynaphthalene,

1-iodo-8-methoxynaphthalene,

8-methoxynaphth-2-yladamant-1'-yl alcohols,

8-methoxynaphth-2-yladamant-1'-yl ketone,

methoxy(8-methoxynaphth-1-yl)methylene adamantane,

methoxy(8-acetoxynaphth-1-yl)methylene adamantane, and

3-(2'-spiroadamantane)-4-methoxy-4-(8"-acetoxy)naphth-1'-yl-

1,2-dioxetane.

And by synthesizing:

methoxy(7-disodiumphosphoryloxynaphth-2-yl)-methylene

adamantane, and

methoxy(8-disodiumphosphoryloxynaphth-1-yl)-methylene

adamantane,

by phosphorylating the corresponding methoxynaphthyl compounds in

the manner disclosed in the aforementioned copending Edwards

application and then photooxygenating these phosphate esters as

described in Example I above, the corresponding 1,2-dioxetanes:

3-(2'-spiroadamantane)-4-methoxy-4-(7"-disodiumphosphoryl-

oxy)naphth-2'-yl-1,2-dioxetane, and

3-(2'-spiroadamantane)-4-methoxy-4-(8"-disodiumphosphoryl-

oxy)naphth-1'-yl-1,2-dioxetane,

are obtained.

EXAMPLE II

Forty μl of a 5 x 10^{-3}M acetonitrile solution of

chromatographed 3-(2'-spiroadamantane)-4-methoxy-4-(7"-

acetoxy)naphth-2'-yl-1,2-dioxetane were dissolved in 2 ml of

phosphate buffer (pH=8) containing 0.1% bovine serum albumin

(BSA) in a cuvett. Eight μl of a carboxylesterase suspension

(Sigma No. E3128) were added to the cuvett and the resulting light emission was recorded on a Spex Fluorolog Fluorimeter by accumulating 5 successive scans (400-700 nm.). A plot of this emission's intensity vs. wavelength, showing a slight blue shift in the presence of BSA as compared to hydroxide ion-induced decomposition in water (530 nm. v. 555 nm.), is attached as FIG. 1 (compare with FIG. 2, Curve A).

EXAMPLE III

Methoxy(6-acetoxynaphth-2-yl)methylene adamantane was synthesized as described in the aforementioned copending Edwards application. This compound was then photooxygenated in the same manner as described in Example I above. The resulting 3-(2'-spiroadamantane)-4-methoxy-4-(6"-acetoxy)naphth-2'-yl-1,2-dioxetane, after column chromatography, exhibited the following NMR spectrum (CDCl$_3$, p.p.m.): 0.95-2.1 (12H, m); 2.25 (1H, s); 2.38 (3H, s); 3.12 (1H, s); 3.28 (3H, s); 7.28-8.15 (6H, m). One hundred μl of a 5 X 10^{-3}M solution of this dioxetane in acetonitrile was placed in a cuvett, followed quickly by the addition of 2 ml 75 mM NaOH solution. The slightly cloudy solution was placed in a Spex Fluorolog Fluorometer and light emission accumulated over 5 successive scans from 400 to 700 nm. at room temperature. This experiment was then repeated exactly using a 5 X 10^{-3}M acetonitrile solution of the corresponding 2,7-substituted dioxetane as synthesized in Example I. The chemiluminescent emission spectra of the two dioxetanes were plotted simultaneously on intensity vs. wavelength axes. The

emission from the 2,7-isomer in this predominately aqueous
experiment occurred at 555 nm. while the less intense emission
from the 2,6-isomer occurred at 459 nm. in the same medium.
This plot is attached as FIG. 2 ("A" is the curve for the 2,7-
5 naphthalene dioxetane isomer).

An identical set of experiments was then performed comparing
the emission from 3-(2'-spiroadamantane-4-methoxy-4-(7"-
acetoxy)naphth-2'-yl-1,2-dioxetane and 3-(2'-spiroadamantane)-4-
methoxy-4-(3"-acetoxy)phenyl-1,2-dioxetane (synthesized as
10 described in the aforementioned copending Edwards application) as
5×10^{-3} M solutions in CH_3CN. The comparative plot is shown in
FIG. 3 ("A" is the curve for the 2,7-naphthalene dioxetane).

The naphthalene based system again emits light at 555 nm.,
while the acetoxyphenyl dioxetane does so as 473 nm. with
15 similar intensity.

EXAMPLE IV

One hundred μl of a 5×10^{-3} M solution of 3-(2'-
spiroadamantane)-4-methoxy-4-(6"-acetoxy)napth-2'-yl-1,2-
dioxetane in CH_3CN was placed in a cuvett, followed by the rapid
20 addition of 2 ml 75 mM NaOH in 20% CH_3CN/H_2O. The clear solution
was placed in a Spex Fluorolog Fluorometer at room temperature
and the resulting light emission recorded by accumulating 5
successive scans (400-700 nm.). This process was repeated for
an equimolar CH_3CN solution of 3-(2'-spiroadamantane)-4-methoxy-
25 4-(7"-acetoxy)napth-2'-yl-1,2-dioxetane. FIG. 4 shows the
intensity vs. wavelength plot for the chemiluminescence produced

via saponification in each isomer. The 2,6-isomer displays its emission maximum at 436 nm. while the more intense emission of the 2,7-isomer occurs at 543 nm. in the mixed solvent ("A" is the curve for the 2,7-naphthalene dioxetane).

5

EXAMPLE V

The procedure of Example IV was followed exactly, except that 2 ml of 75 mM NaOMe (Aldrich Chemical Co.) in acetonitrile containing the minimum methanol required to maintain homogeneity was used as the non-aqueous solvent. The emission plots obtained

10   for the two isomeric acetoxy naphthalene dioxetanes (2,6 and 2,7) under these non-aqueous conditions are shown in FIG. 5. The 2,7 isomer emits light at 552 nm. while the 2,6 isomer emits at 430 nm. ("A" is the curve for the 2,7-naphthalene dioxetane).

EXAMPLE VI

15   Following the procedure of Example V, 100 μl of 5 X 10^{-3}M solutions of 3-(2'-spiroadamantane)-4-methoxy-4-(3"-acetoxy)phenyl -1,2-dioxetane and 3-(2'-spiroadamantane)-4-methoxy-4-(7"-acetoxy)naphth-2'-yl-1,2-dioxetane were independently saponified in the fluorometer with 2 ml 75 mM NaOMe

20   (Aldrich) at room temperature. After 5 scans, the light emission from 400 to 700 nm. in each case was plotted, and is attached as FIG. 6. The 2,7-naphthalene dioxetane (Curve "A") still emits in the green region of the visible spectrum (554 nm.).

EXAMPLE VII

Twenty μl of a 5 x 10^{-3}M solution of 3-(2'-spiroadamantane)-4-methoxy-4-(6"-acetoxy)naphth-2'-yl-1,2-dioxetane in CH_3CN was placed in a glass tube inside a Turner Model 20E Luminometer at
5 room temperature. Four hundred fifty μl of a 75 mM solution of NaOH in 20% CH_3CN/80% H_2O was injected manually. Light emission essentially ceased after 3.5 minutes (FIG. 7). The experiment was performed in triplicate and the procedure was then repeated for an equimolar solution of the isomeric 2,7-acetoxynaphthalene
10 dioxetane. In the latter case, the longer duration (beyond 30 minutes) and the larger total integral of light energy are displayed in FIG. 8. The extended glow of the 2,7-substituted dioxetane provides greater sensitivity, simplicity of light detection and flexibility of design in immunoassays and DNA probe
15 technology.

EXAMPLE VIII

[Synthesis of 3-(2'-spiroadamantane)-4-methoxy-4-
(6"-acetoxy)naphth-1'-yl-1,2-Dioxetane]

1-Amino-6-hydroxynaphthalene. Sodium bisulfite (17.96 g,
20 0.173 mol) was added in portions to a solution of 1,6-dihydroxynaphthalene (25.14 g, 0.157 mol) in 30% NH_4OH (200 ml) at 0°C with stirring. The reaction mixture was then heated in a steel bomb at 150°C for 3 hours to induce α-amination. After cooling to room temperature, the solution was acidified with

concentrated HCl and partitioned with EtOAc/H_2O, forcing the naphthylamine hydrochloride into the aqueous layer. The aqueous layer was neutralized with NaOH pellets to free the desired amine, partitioned with EtOAc, washing the aqueous layer once,

5   and the combined EtOAc layers were dried over Na_2SO_4, decanted and evaporated, yielding dark brown crystals of 1-amino-6-hydroxynaphthalene [16.75 g, 66.3%, m.p. 180°C, dec.; literature value is m.p. 186.8°C, JACS, 51 (1929) 1766.].

IR (Nujol, cm^{-1}): 3353, 3387, 1600, 1574, 1303, 1271, 1228

10   1-Amino-6-methoxynaphthalene. 1-Amino-6-hydroxynaphthalene (16.75 g, 0.105 mol) was added in portions to hexane-rinsed NaH (60% 5.13 g, 0.128 mol) in dry DMF (150 ml) at 0°C. The solution was stirred under argon for 10 minutes until hydrogen evolution had ceased, indicating complete sodium naphthoate formation. O-

15 - methylation proceeded via dropwise addition of dimethyl sulfate (10 ml, 0.106 mol) at 0°C until TLC showed consumption of all starting naphthol. The reaction was carefully quenched with concentrated HCl to pH 1 and partitioned between ether and H_2O. After washing the ether layer with 6N HCl, the combined aqueous

20   layers, containing the desired naphthylamine hydrochloride, were neutralized with NaOH pellets, partitioned with EtOAc and washed twice. The EtOAc layers were dried over Na_2SO_4, concentrated by rotary evaporator and the residue was pumped under high vacuum while warming to remove DMF. The crude product was

25   chromatographed on coarse silica gel, eluting with 20% EtOAc/hexanes. Dissolution of the oil in MeOH/H_2O yielded 16.53 g (90.7%) of crystalline 1-amino-6-methoxynaphthalene, m.p. 64°C (Literature value is m.p. 74°C, but has also been

reported as 64°C [J.Org.Chem., 14 (1949) 352).]

IR (CHCl$_3$, cm^{-1}): 3468, 3445, 3393, 3000, 1707, 1623, 1447, 1362, 1271, 1225

NMR (CDCl$_3$, ppm): 3.89 (3H, s); 4.05 (2H, br s); 6.62 (1H, d, J=7.03 Hz); 7.11 (2H, m); 7.22 (1H, d); 7.24 (1H, d); 7.69 (1H, d, J=9.96 Hz)

1-Iodo-6-methoxynaphthalene. Diazotiation of 1-amino-6-methoxynaphthalene (16.53 g, 0.096 mol) in an HOAc (acetic acid) concentrated HCl/H$_2$O mixture (9 ml/24 ml/90 ml) proceeded with dropwise addition of NaNO$_2$ (7.0 g, 0.101 mol) dissolved in 20 ml of H$_2$O at 0°C. The reaction mixture was stirred for 1 hour at low temperature and then converted to the N,N-diethyltriazene upon addition of K$_2$CO$_3$ (33 g, 0.239 mol, dissolved in 20 ml H$_2$O and diethylamine (15 ml, 0.145 mol). After adding ethyl ether (50 ml) to dissolve the newly formed naphthalene triazene, followed by stirring for 40 minutes, the reaction solution was partitioned with CH$_3$CN/EtOAc 1:1. The aqueous phase was drawn off and washed well with CH$_3$CN/EtOAc. The combined organic layers were dried and evaporated under reduced pressure with low heat to give the crude triazene as an orange oil, which was used immediately, without purification, in the next reaction.

1-(3',3-Diethyl)triazo-6-methoxynaphthalene, dissolved in 120 ml dry CH$_3$CN, was added dropwise (1 drop/4 sec) to a slurry of ground KI (35 g, 0.211 mol), Amberlyst XN -1010 resin (30 g) and ground molecular sieves in dry DMF/CH$_3$CN (50 ml/150 ml) at 70°C overnight. When TLC indicated complete iodide formation, the reaction mixture was cooled, decanted into a separatory funnel and the resin was rinsed with three CH$_3$CN washes. The

CH$_3$CN layers were combined, evaporated to a small volume and partitioned with hexanes as necessary to thoroughly extract the desired iodonaphthalene from the CH$_3$CN layer. The hexanes solution was evaporated and quickly passed through coarse silica

5   gel, eluting with hexanes, to yield 7.245 g (27%) of 1-iodo-6-methoxynaphthalene as a yellow-orange oil which slowly crystallized upon refrigeration.

IR (CHCl$_3$, cm^{-1}): 1622, 1593, 1501, 1468, 1372, 1355, 1234, 1171, 1123, 1032, 955, 846, 823, 652

10   NMR (CDCl$_3$, ppm): 3.85 (3H, s); 6.99 (1H, d, J=2.55 Hz); 7.02 (1H, m); 7.12 (1H, dd, J=9.28, 2.55 Hz); 7.63 (1H, d, J=8.09 Hz); 7.82 (1H, dd, J=7.32, 1.09 Hz); 7.90 (1H, d, J=9.28 Hz)

6-Methoxynaphth-1-yladamant-1'-yl alcohol. Lithiation of 1-iodo-6-methoxynaphthalene (38 mg, 0.134 mmol) in 1 ml anhydrous

15   ether proceeded cleanly upon addition of n-BuLi (1.6M, 250 µl) at -65°C with subsequent warming to -15°C under an argon atmosphere. Dropwise addition of adamantane-2-carboxaldehyde (41 mg, 0.25 mmol) dissolved in 1 ml anhydrous ether to the naphthyllithium solution immediately yielded the coupling product.

20   The reaction was quenched upon partitioning between EtOAc and H$_2$O, the EtOAc was dried through a Na$_2$SO$_4$ column and purified on a silica gel column, eluting with 5-20% EtOAc/hexanes, to yield 5.5 mg of alcohol for spectral characterization.

IR (CHCl$_3$, cm^{-1}): 3603, 2904, 2847, 1624, 1601, 1512, 1473,

25   1453, 1434, 1377, 1172, 1044, 1020, 851, 831

6-Methoxynaphthyladamant-2'-yl ketone. The 6-methoxynaphth-1-yladamant-2'-yl alcohols (5.5 mg, 0.017 mmol) were dissolved in 1 ml acetone and oxidized to the aromatic ketone with Jones

reagent at 0°C. After partitioning the solution between
EtOAc/H$_2$O and washing the aqueous layer three times with EtOAc,
the collected organic layers were dried and evaporated to a film.
The crude ketone was purified on analytical TLC, eluting the plate

5   with 10% EtOAc/hexanes, to give 4.4 mg (80%) of the desired ketone.

IR (CHCl$_3$, cm^{-1}): 3021, 2905, 2845, 1674 (C=O), 1623, 1593,
1508, 1471, 1453, 1433, 1374, 1173, 1095, 1044, 843

NMR (CDCl$_3$, ppm): 1.48-1.93 (12H); 2.29 (2H, br s);
3.28 (1H, br s); 3.84 (3H, s); 7.07 (1H, d, J=2.66 Hz); 7.12 (1H,

10   dd, J=9.34, 2.6 Hz); 7.33 (1H, m); 7.38 (11h, DD, j=7.11, 1.35
hZ); 772 (1h, D, j=7.87 hZ); 8.09 (1h, D, j=9.27 hZ)

An alternative synthesis of the 1,6-naphthalene regioisomer
is described below, resulting in 1-bromo-6-methoxynaphthalene.
As described above for 1-iodo-6-methoxynaphthalene, the

15   naphthalene bromide was also coupled with adamantane-2-
carboxaldehyde and oxidized to the 6-methoxynaphth-1-yladamant-
1'-yl ketone. The nmr data for the naphthyladamantyl ketones,
derived independently from the iodo- and bromonaphthalene
derivatives are virtually superimposable, thus confirming the

20   regiochemistry of the naphthyl halides as being identical.

1-Bromo-6-methoxynaphthalene. 6-Methoxy-1-tetralone (14.42
g, 0.082 mol) dissolved in EtOAc (100 ml) was added to a solution
of freshly made PBr$_5$ (38.8 g, 0.090 mol) and Na$_2$SO$_3$ (5.9 g, 0.047
mol) in 100 ml EtOAc, with stirring, at room temperature. The

25   reaction mixture was gently refluxed until analytical TLC
indicated no change in the product mixture 23 hours), at which
time the solution was cooled and portioned between EtOAc and H$_2$O.
The EtOAc layer was then dried over Na$_2$SO$_4$, decanted and

evaporated to yield crude 1-bromo-3,4-dihydro-6-methoxynaphthalene.

The dihydronaphthalene was aromatized by refluxing with p-chloranil (12.76 g, 0.052 mol) and 0.5 ml HOAc in toluene (160 ml) for 68 hours. Upon complete oxidation, the cooled mixture was portioned between EtOAc/CH$_3$CN 11:1 and 1M NaOH. The organic layer was washed with dilute NaOH twice and the combined aqueous layers were, in turn, washed with EtOAc once. The organic layers were then dried over Na$_2$SO$_4$, decanted and evaporated under reduced pressure to a brown oil. Chromatography on coarse silica gel (60-200 mesh), with hexanes as the eluent, gave 10.3 g (53% in 2 steps) of the desired 1-bromo-6-methoxynaphthalene.

IR (CHCl$_3$, cm^{-1}): 3003, 2958, 2937, 1622, 1594, 1564, 1503, 1468, 1423, 1373, 1359, 1263, 1233, 1170, 1125, 1033, 960, 918, 844, 823, 661

NMR (CDCl$_3$, ppm): 3.86 (3H, s); 7.05 (1H, d, J=2.44 Hz); 7.15-7.21 (2H, m); 7.57 (1H, d, J=6.59 Hz); 7.60 (1H, d, J=8.11 Hz); 8.06 (1H, d, J=9.28 Hz)

6-Methoxynaphth-1-yladamant-1'-yl alcohol. 1-Bromo-6-methoxynaphthalene (92.3 mg, 0.389 mmol) dissolved in 2 ml anhydrous ether was lithiated upon addition of n-BuLi (1.6M, 365 µl) at -60°C under argon. After allowing the reaction to stir for 10 minutes, adamantane-2-carboxaldehyde (77 mg, 0.467 mmol) in 2 ml ether was added dropwise to effect coupling. The reaction mixture was warmed to room temperature, quenched by partitioning with water, and the collected ether layer was dried through a Na$_2$SO$_4$ column and evaporated. The crude alcohol mixture was quickly flashed through a silica gel column, eluting

with 10% EtOAc/hexanes to give a purified mixture for spectral analysis. No yield was recorded.

IR(CHCl$_3$, cm^{-1}): 3593, 3003, 2904, 2893, 1623, 1597, 1511, 1470, 1452, 1433, 1373, 1263, 1170, 1133, 1043, 1023, 846, 828

6-Methoxynaphth-1-yladamant-1'-yl ketone. The mixture of 6-methoxynaphth-1-yladamant-1'-yl alcohols was dissolved in 2 ml acetone and oxidized at 0°C with dropwise addition of Jones reagent. Workup by partitioning the solution between EtOAc/H$_2$O, evaporating and chromatographing the crude product on silica gel (10% EtOAc/hexanes) afforded pure 6-methoxynaphth-1-yladamant-1'-yl ketone for spectral analysis. No yield was recorded.

IR (CHCl$_3$, cm^{-1}): 3005, 2905, 2845, 1675 (C=O), 1624, 1595, 1509, 1471, 1455, 1435, 1376, 1260, 1245, 1175, 1096, 1044, 950, 845, 814

NMR (CDCl$_3$, ppm): 1.50-1.96 (12H); 2.30 (2H, overlapping singlets); 3.30 (1H, s); 3.86 (3H, s); 7.09 (1H, d, J=2.5 Hz); 7.14 (1H, dd, J=9.28, 2.7 Hz); 7.33-7.42 (2H, m); 7.74 (1H, d, J=8.05 Hz); 8.11 (1H, d, J=9.52 Hz)

By proceeding from this point on in the synthesis in the manner described in Example I above, i.e., converting 6-methoxynaphth-1-yladamant-1'-yl ketone to the methyl enol ether, deprotecting with sodium ethanethiolate in dry DMF to give the naphthol followed by in situ acylation with acetic anhydride/pyridine to give methoxy-(6-acetoxynaphth-1'-yl) methylene adamantane, and then photoxygenating this compound's ethylenic double bond, 3-(2'-spiroadamantane)-4-methoxy-4-(6"-acetoxy)naphth-1'-yl-1,2-dioxetane is obtained.

46

EXAMPLE IX

[Synthesis of 3-(2'-spiroadamantane)-4-methoxy-4-(5"-acetoxy)naphth-2'-yl-1,2-dioxetane and 3-(2'-spiroadamantane)-4-methoxy-4-(5"-phosphoryloxy)naphth-2'-yl-1,2-dioxetane].

2-Bromo-5-methoxynaphthalene. Under an argon atmosphere, 60% sodium hydride (1.4g, 0.035 mol) is washed 3 times with hexanes. Sieve dried (3A) DMF (40 ml) is added and the suspension is cooled to 0°C while 6-bromo-1-naphthol (5g., 0.032 mol; Hodgson et al, J.Chem.Soc., 8, 1944) is added with stirring. After hydrogen evolution ceases, dimethyl sulfate (3.4 ml, 0.036 mol) is added dropwise. Stirring is continued in the cold for 8 hours. The reaction mixture is diluted with 250 ml ice cold 0.05N NaOH to yield a gummy precipitate. The aqueous layer is decanted and the residue is triturated several times with dilute NaOH. Plug chromatography on silica gel (15% ethyl acetate-hexanes) affords a good yield of the bromo ether.

R and S 5-methoxynaphth-2'-yl-adamanat-2'-yl alcohol. 2-Bromo-5-methoxynaphthalene (500 mg.; 2.1 mmol) is dissolved in 25 ml anhydrous ether under argon. The solution is cooled to 0°C and treated with a solution of n-BuLi (1.6M in hexanes, 2 ml) via syringe. The resulting solution is stirred in the cold for 15 minutes prior to the addition of a solution of adamantane-2-carboxaldehyde (416 mg., 2.5 mmol) in 20 ml anhydrous ether. The mixture is slowly warmed to room temperature and quenched with 50 ml 1% NH₄Cl solution. The ether layer is removed, extracted again with water, and dried over Na₂SO₄. The solvent is stripped to afford an oil which is flash chromatographed on silica gel

with 10% ethyl acetate-hexanes as eluant. The fractions containing the lower Rf isomeric alcohols are combined and concentrated to yield product in moderate yield.

5-Methoxynaphth-2-yladamant-2'-yl ketone. A solution of the
5   5-methoxynaphth-2-yl-adamant-2'-yl alcohols (700 mg., 2.17 mmol) in 20 ml acetone is cooled to 0°C and treated dropwise with Jones reagent (23 ml conc. H_SO_4 and 26.9g. CrO_3 and water to 100 ml) while stirring vigorously. The rapid reaction is monitored by TLC until the lower Rf starting material is replaced by the less
10 polar ketonic product. The greenish yellow mixture is partitioned between 60 ml ethyl acetate and water. The aqueous layer, which contains some solid, is extracted three times with ethyl acetate (20 ml). The combined organic fractions are washed with saturated $NaHCO_3$ solution, dried over Na_2SO_4, and stripped
15 to a light yellow oil which exhibits the characteristic carbonyl stretch in the infrared spectrum. The crude product is used directly for conversion to the enol ether.

Methoxy (5-methoxynaphth-2-yl) methylene adamantane. A solution of the crude 5-methoxynaphth-2-yladamant-2'-yl ketone
20 (1g., 3.1 mmol) in 35 ml sieve dried (3A) DMSO at 50°C, is treated with potassium tert-butoxide (0.7g., 6.3 mmol) under a leisurely flow of argon. The colored solution of the enolate anion is stirred for 10 minutes. The solution becomes decolorized during a 15 minute dropwise addition of dimethyl
25 sulfate (595 μl, 6.3 mmol). After cooling to room temperature the solution is stirred overnight and partitioned between ethyl acetate and water. The organic layer is washed three times with water and dried over anhydrous K_2CO_3. Concentration in vacuuo

yields an oil which is immediately plug chromatographed on silica
gel with 5% ethyl acetate hexanes to yield the enol ether in
good yield. TLC analysis in the same solvent system shows that
a trace of the more polar ketonic starting material is present as
5   a contaminant.

Methoxy-5(5-hydroxynaphth-2-yl)methylene adamantane. Under
an argon atmosphere, 60% sodium hydride (200 mg, 5 mmol) is
washed twice with hexanes. Excess solvent is blown off and
sieve-dried DMF (15 ml) is added. The resulting slurry is cooled
10   to 0°C with stirring. Ethanethiol (370 μl, 5 mmol) is added
dropwise via syringe. After 10 minutes the mixture is warmed to
room temperature and stirred until hydrogen evolution abates.
Methoxy-(5-methoxynaphth-2-yl)methylene adamantane (750 mg.,
2.24 mmol) in 10 ml DMF is added all at once and the resulting
15   solution is refluxed for 3.5 hours. DMF is removed in vacuuo and
the residue is partitioned between 5% NH₄Cl and 25 ml ethyl
acetate. The aqueous layer is extracted again with another
equivalent volume of ethyl acetate. The combined organic layers
are washed with 2 X 30 ml water, 1 X 30 ml saturated NaCl, and
20   dried quickly over sodium sulfate. TLC (Whatman K5F, 20% ethyl
acetate: CH₂Cl₂) shows the absence of starting material on a
single low Rf product. The solvent is stripped and the residue
recrystallized from CH₃CN to provide the naphthol in good yield.

3-(2'-Spiroadamantane)-4-methoxy-4-(5"-acetoxy)naphth-2'-yl-
25   1,2-dioxetane. By proceeding from this point on in the
synthesis in the manner described in Example I above, i.e.,
acetylating methoxy-(5-hydroxynaphth-2'-yl)methylene adamantane
with acetic anhydride/pyridine to give methoxy-(5-acetoxynaphth-

2'-yl)methylene adamantane, and then photooxygenating this compound's ethylenic double bond, 3-(2'-spiroadamantane)-4-methoxy-4-(5"-acetoxy)naphth-2'-yl-1,2-dioxetane is obtained.

3-(2'-Spiroadamantane)-4-methoxy-4-(5"-phosphoryloxy)naphth-2'-yl-1,2-dioxetane. Phosphorylation of methoxy-(5-hydroxynaphth-2'-yl)methylene adamantane via the 2-chloro-2-oxo-1,3-dioxa phospholane method described in the aforementioned copending Edwards application to give methoxy-(5-phosphoryl-oxynaphth-2'-yl)methylene adamantane, and then photooxygenating this compound's ethylenic double bond, gives 3-(2'-spiroadamantane)-4-methoxy-4-(5"-phosphoryloxy)naphth-2'-yl-1,2-dioxetane.

EXAMPLE X

A dual channel assay for Human Chorionic Gonadotropins (β-chain), β-HCG, and Human Luteinizing Hormones, HLH, is carried out as follows:

MATERIALS:

A round nylon membrane (approximately 1 inch in diameter) containing two sets of covalently immobilized capture monoclonal antibodies is used, one set for β-HCG available from Medix Biotech, Anti HCG, Cat. No. H298-01, and the second for HLH also available from Medix Biotech, Anti LH, Cat. No. L461-09. This nylon membrane is stored in a foil pouch until used.

Mouse monoclonal anti β-HCG available from Medix Biotech, Cat. No. H298-12, is conjugated with alkaline phosphatase using the glutaraldehyde coupling procedure [Voller, A., et. al., _Bull. World Health Org._, _53_, 55 (1976)] and is used as a detection

antibody for β-HCG.

Mouse monoclonal anti HLH available from Medix Biotech, Cat. No. L461-03, is conjugated to carboxylesterase also using the glutaraldehyde coupling procedure referenced above, and is used as a detection antibody for HLH.

The substrate buffer solution used contains 0.05M carbonate, 1mM $MgCl_2$, 0.1% by weight BSA (pH = 0.5), and 3-(2'-spiroadamantane)-4-methoxy-4(3'-phosphoryloxy)phenyl-1,2-dioxetane disodium salt (50 μg/ml) and 3-(2'-spiroadamantane)-4-methoxy-4-(7"-acetoxy)naphth-2'-yl-1,2-dioxetane (50 μg/ml) as the chemiluminescent substrates.

The wash buffer used contains 0.05M carbonate, 1mM $MgCl_2$ and 2% by weight BSA (pH = 9.5).

ASSAY PROCEDURE:

Five drops of a previously collected urine sample are placed onto the center of the assay membrane and allowed to soak into the membrane. Next, five drops of a solution containing β-HCG and HLH conjugated detection antibodies at a concentration of 0.01 millimolar are added to the assay membrane. The liquid is allowed to soak in for at least one minute. Six drops of the wash buffer are slowly added and allowed to soak in and drain for 30 to 60 seconds. Then, five drops of the buffer solution containing chemiluminescent substrates are added and allowed to soak in for approximately one minute.

The assay membrane is placed in a camera luminometer device equipped with pre-exposed calibration scales for β-HCG and LH.

The chemiluminescent light emission, generated as a function of the enzymes, alkaline phosphatase and carboxyl esterase, is

imaged through a mask containing two narrow band pass filters (approximately 1cm in diameter). Kodak Wratten Gelatin Filter No. 115 is used to image the green emission from the naphth-2'-yl-1,2-dioxetane substrate, and Kodak Wratten Filter No. 47B is

5   used to isolate the blue emission from the phenyl dioxetane.

The relative levels of β-HCG and HLH present in the sample are determined by a comparison of the appropriate imaged spot brightness with relevant calibration scales.

EXAMPLE XI

10   A three-channel analysis for Herpes Simplex Virus, (HSV), Cytomegalovirus, (CMV), and Human Papiloma Virus, (HPV) is carried out as follows:

MATERIALS:

"Gene Screen Plus", a positively charged nylon membrane

15 (Dupont NEN Products) is used for hybridization.

The following buffers are used for the assay:

HSV DNA PROBE ASSAY

Materials and Buffers:

Membrane: Gene Screen Plus membrane.

20 Buffers: Denaturation Buffer, 0.5 M NaOH

Neutralization Buffer, 0.4M NaH_2PO_4 (pH = 2.0)

Loading Buffer, 1 part Denaturation Buffer

1 part Neutralization Buffer

Membrane Wetting Buffer 0.4M Tris (pH = 7.5)

<u>Membrane Prehybridization Buffer</u>:

<u>Substance</u>	<u>Final Concentration</u>
0.5 ml 100 X Denhardt's solution	5 X
0.5 ml 10% SDS	0.5%
2.5 ML 20 x SSPE	5 X
2.0 mg denatured, sonicated salmon sperm DNA	200 µg/ml

<u>ddH$_2$O</u>
10ml

<u>Membrane Hybridization Buffer</u>:

<u>Substance</u>	<u>Final Concentration</u>
0.5 ml 100 X Denhardt's solution	5 X
0.5 ml 10% SDS	0.5%
2.5 ml 20 X SSPE	5 X
2.0 mg salmon sperm DNA	200 µg/ml
2.0 ml 50% Dextran sulfate	10%
___ ddH$_2$O	
10 ml	

<u>Wash Buffer I</u>:

1 X SSPE/0.1% SDS
   20ml 20 X SSPE
    4ml 10% SDS
   <u>376ml</u> ddH$_2$O
   400ml

<u>Wash Buffer II</u>:

0.1 X SSPE/0.1% SDS preheated
to wash temperature indicated on
Technical Data Sheet.
    2ml 20 X SSPE
    4ml 20% SDS
    <u>394ml</u> ddh$_2$O
    400ml (heated)

<u>Wash Buffer III</u>:

0.1 X SSPE/0.1% SDS
  2 ml20 X SSPE
  4 ml10% SDS
<u>394ml</u> ddH$_O$
400ml

Wash Buffer IV:

    3mM Tris-HCl (pH 9.5)
        0.6ml 1 M Trizma Base
    199.4ml ddH$_2$O
    200.0ml

Wash Buffer V:

    0.1 M Trizma HCl pH 6.0

    100 X Denhart's solution

Preparation of 100 X Denhart's solution (for 100 mls):

Polyvinylpyrrolidone (2g; mol. wt. 40K; PVP-40) and 2g ficoll
are dissolved at a temperature greater than 65°C but less than
boiling. The solution is cooled to approximately 40°C, 2g BSA
are added and the solution is brought up to the final volume of
100 ml with ddH$_2$O, aliquoted and stored at -20°C. BSA is easily
denatured and in combination with PVP and ficoll it will not go
into solution at all if it is not cooled down to -40°C. Hence,
the solution is not heated over 40°C when thawing for use.

Preparation of 20 X SSC solution:

    20 X SSC (for 100 ml)

    3.0 M Sodium Chloride   17.4g

    0.3 M Sodium Citrate   8.8g

The volume is brought to 100 ml and the solution
filtered through a 0.45 μm nitrocellulose filter and stored at
room temperature.

<u>Preparation of 20 X SSPE solution</u>:

         20 X SSPE pH 7.4 (for 1 liter)

         3.6 M NaCl

         200 mM Sodium phosphate 23 g dibasic, 5.92g monobasic

5   20 mM EDTA   7.44g

These materials are dissolved and adjusted to a pH of 7.4 with 5N NaOH. The volume is then brought to 1 liter and the solution filtered through a 0.45 μm nitrocellulose filter.

10   <u>1 X TE</u>:

         1 X TE buffer   10 mM Tris (pH 7.)

                               1 mM EDTA

                               Autoclave

         The substrate buffer solution used contains 0.05M

15   carbonate, 1 mM $MgCl_2$, 0.1% by weight BSA (Ph = 9.5), and 3-(2'-spiroadamantane)-4-methoxy-4-(3'-phosphoryloxy)phenyl-1,2-dioxetane disodium salt (50 mg/ml), 3-(2'-spiroadamantane)-4-methoxy-4-(7"-acetoxy)naphth-2'-yl-1,2-dioxetane (50 mg/ml) and 3-(2'-spiroadamantane)-4-methoxy-4-(5"-β-galactosyloxy)naphth-2'-

20   yl-1,2-dioxetane (50 mg/ml) as the chemiluminescent substrates.

<u>ASSAY PROCEDURE</u>:

         Samples (50 μl) containing DNA are denatured by incubation for 10 minutes at room temperature in 200 μl of Denaturation Buffer. Ice cold Neutralization Buffer (250 μl) is then added,

ana the samples placed on ice.

Nylon membrane is soaked for 15 minutes in Wetting Buffer and then inserted into a vacuum manifold device producing 2 cm diameter spots. Loading Buffer, (200 μl) is then aspirated

5   through each well. The denatured DNA samples are then added to each well and aspirated through the membrane. The manifold is then disassembled and the DNA fixed to the membrane using a UV Transilluminator for 5 minutes. The membrane is then incubated in Prehybridization Buffer at 70°C for 1 hour.

10   Dots of membrane from the region in which the sample DNA is applied are punched out and inserted into tubes for the remaining steps of the assay. The following enzyme labeled probes are used: probe for HSV labeled with alkaline phosphatase; probe for HPV labeled with β-galactosidase; probe

15   for CMV labeled with carboxylesterase.

The enzyme labeled probes (50 ng of each probe in 200 μl of Hybridization Buffer per tube) are added to each tube and incubated for 2 hours at 70°C. The Hybridization Buffer is then removed and 400 μl of Wash Buffer I added. The tubes are then

20   agitated for 10 minutes at room temperature. Washing is continued by first washing with 400 μl of Wash Buffer II at 50°C for 30 minutes; then with 400 μl of Wash Buffer III at room temperature for 10 minutes; and then with 200 μl of Wash Buffer IV at room temperature.

25   The membrane is subsequently rinsed with Wash Buffer V at pH 6.0 and placed on a piece of transparent Mylar polyester film. Then, 200 μl of the Substrate Buffer is added and allowed to soak in.

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The assay membrane is placed in a camera luminometer device equipped with pre-exposed calibration scales for HSV, HPV and CMV.

5   The chemiluminescent light emission generated as a function of the enzymes -- alkaline phosphatase, carboxyl esterase and β-galactosidase -- is imaged through a mask containing three narrow bandpass filters (approximately 1cm in diameter). Kodak Wratten Gelatin Filter No. 15 is used to image green emission from the naphthyl acetate dioxetane substrate, Kodak Wratten Filter No.

10   47B is used to isolate blue emission from the phenyl phosphate dioxetane, and Kodak Wratten Gelatin Filter No. 24 is used to image the orange emission from the naphthyl galactose dioxetane.

The relative levels of HSV, HPV and CMV present in the sample are determined by a comparison of the appropriate image

15   brightness with relevant calibration scales.

The above discussion of this invention is directed primarily to preferred embodiments and practices thereof. It will be readily apparent to those skilled in the art that further changes and modifications in the actual implementation of the concepts

20   described herein can easily be made without departing from the spirit and scope of the invention as defined by the following claims.

WE CLAIM:

1. A chemiluminescent 1,2-dioxetane compound having the formula:
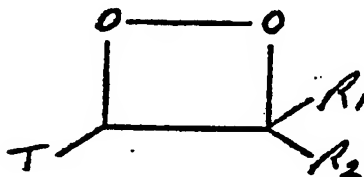
wherein R_1 is hydrogen, or a bond when R_2 is a substituent bound
to the dioxetane ring through a spiro linkage, or an organic
substituent that does not interfere with the production of light
but satisfies the valence of the dioxetane ring carbon atom to
which it is attached; R_2 is a fused polycyclic ring-containing
fluorophore moiety bound to the dioxetane ring through a single
bond or a spiro linkage and having a labile ring substituent
containing a bond which, when cleaved, renders the polycyclic
moiety electron-rich to in turn render the dioxetane compound
decomposable to emit light, the labile ring substituent's point
of attachment to the fused polycyclic moiety, in relation to the
fused polycyclic moiety's point of attachment to the dioxetane
ring, being such that the total number of ring atoms separating
these points of attachment, including the ring atoms at these
points of attachment, is an odd whole number; and T is a
stabilizing group that prevents the dioxetane compound from
decomposing before the labile ring substituent's bond is cleaved.

2. A chemiluminescent 1,2-dioxetane compound as recited in
claim 1 in which the odd whole number is 5 or greater.

3. A chemiluminescent 1,2-dioxetane compound as recited in

claim 2 in which the fused polycyclic moiety is the residue of a fused polycyclic aromatic hydrocarbon ring fluorophoric compound containing from 9 to about 30 ring carbon atoms, inclusive.

4. A chemiluminescent 1,2-dioxetane compound as recited in claim 3 in which the residue of a fused polycyclic aromatic hydrocarbon ring fluorophoric compound is a naphthalene residue.

5. A chemiluminescent 1,2-dioxetane compound as recited in claim 3 in which the residue of a fused polycyclic aromatic hydrocarbon ring fluorophoric compound is an anthracene residue.

6. A chemiluminescent 1,2-dioxetane compound as recited in claim 2 in which the fused polycyclic moiety is the residue of a less than fully aromatic fused polycyclic hydrocarbon ring fluorophoric compound containing from 10 to about 30 carbon atoms, inclusive.

7. A chemiluminescent 1,2-dioxetane compound as recited in claim 6 in which the residue of a less than fully aromatic fused polycyclic hydrocarbon ring fluorophoric compound is a fluorene residue.

8. A chemiluminescent 1,2-dioxetane compound as recited in claim 2 in which the fused polycyclic moiety is the residue of a fused polycyclic heterocyclic ring fluorophoric compound containing from 9 to about 30 ring atoms, inclusive.

9. A chemiluminescent 1,2-dioxetane compound as recited in claim 8 in which the residue of a fused polycyclic heterocyclic ring fluorophoric compound is a quinoline residue.

10. A chemiluminescent 1,2-dioxetane compound as recited in any one of claims 3-9, inclusive, in which R_1 is a methoxy group, R_2 is bound to the dioxetane ring through a single bond

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and T is an adamant-2-ylidene group.

11. A chemiluminescent 1,2-dioxetane compound as recited in claim 10 in which the labile ring substituent is enzymatically cleavable.

12. A chemiluminescent 1,2-dioxetane compound as recited in claim 11 in which the labile ring substituent is a phosphate ester group.

13. A chemiluminescent 1,2-dioxetane compound as recited in claim 11 in which the labile ring substituent is a galactoside group.

14. A chemiluminescent 1,2-dioxetane compound as recited in claim 10 in which the labile ring substituent is chemically cleavable.

15. A chemiluminescent 1,2-dioxetane compound as recited in claim 14 in which the labile ring substituent is an acetate ester group.

16. A chemiluminesent 1,2-dioxetane compound as recited in claim 14 in which the labile ring substituent is an organic silyloxy group.

17. A chemiluminescent 1,2-dioxetane compound as recited in claim 16 in which the labile ring substituent is a t-butyldimethylsilyloxy group.

18. A 3-(2'-spiroadamantane)-4-methoxy-4-(6"-phosphoryl-oxy)naphth-1'-yl-1,2-dioxetane salt.

19. A 3-(2'-spiroadamantane)-4-methoxy-4-(7"-phosphoryl-oxy)naphth-2'-yl-1,2-dioxetane salt.

20. A 3-(2'-spiroadamantane)-4-methoxy-4-(8"-phosphoryloxy)naphth-1'-yl-1,2-dioxetane salt.

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21. A 3-(2'-spiroadamantane)-4-methoxy-4-(5"-phosphoryloxy)naphth-2'-yl-1,2-dioxetane salt.

22. The disodium salt of a 1,2-dioxetane compound as recited in any one of claims 14-17, inclusive.

23. 3-(2'-spiroadamantane)-4-methoxy-4-(6"-acetoxy)-naphth-1'-yl-1,2-dioxetane.

24. 3-(2'spiroadamantane)-4-methoxy-4-(7"-acetoxy)naphth-2'-yl-1,2-dioxetane.

25. 3-(2'-spiroadamantane)-4-methoxy-4-(8"-acetoxy)naphth-1'-yl-1,2-dioxetane.

26. 3-(2'-spiroadamantane)-4-methoxy-4-(5"-acetoxy)naphth-2'-yl-1,2-dioxetane.

27. A method for generating light which comprises:

(a) providing a chemiluminescent 1,2-dioxetane compound having the formula:

wherein R_1 is hydrogen, or a bond when R_2 is a substituent bound to the dioxetane ring through a spiro linkage, or an organic substituent that does not interfere with the production of light but satisfies the valence of the dioxetane ring carbon atom to which it is attached; R_2 is a fused polycyclic ring-containing fluorophore moiety having a labile ring substituent containing a bond which, when cleaved, renders the fused polycyclic moiety electron-rich to in turn render the dioxetane compound decomposable to emit light, the labile ring substituent's point

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of attachment to the fused polycyclic moiety, in relation to the
fused polycyclic moiety's point of attachment to the dioxetane
ring, being such that the total number of ring atoms separating
these points of attachment, including the ring atoms at these
points of attachment, is an odd whole number; and T is a
stabilizing group that prevents the dioxetane compound from
decomposing before the labile ring substituent's bond is cleaved,
and

(b) decomposing the 1,2-dioxetane compound by cleaving the
labile ring substituent's bond.

28. A method for generating light as recited in claim 27 in
which the labile ring substituent's bond is cleaved by an enzyme.

29. A method for generating light as recited in claim 28 in
which the 1,2-dioxetane compound is a 3-(2'-spiroadamantane)-4-
methoxy-4-(6"-phosphoryloxy)naphth-1'-yl-1,2-dioxetane salt, a 3-
(2'-spiroadamantane)-4-methoxy-4-(7"-phosphoryloxy)naphth-2'-yl-
1,2-dioxetane salt, a 3-(2'-spiroadamantane)-4-methoxy-4-(8"-
phosphoryloxy)naphth-1'-yl-1,2-dioxetane salt or a 3-(2'-
spiroadamantane)-4-methoxy-4-(5"-phosphoryloxy)naphth-2'-yl-1,2-
dioxetane salt.

30. A method for generating light as recited in claim 29 in
which the 1,2-dioxetane salt is a disodium salt.

31. In a process in which light released by the
decomposition of a chemiluminescent chemical compound is detected
to determine the presence, concentration or structure of an
analyte, the improvement comprising carrying out the process in
the presence of at least one chemiluminescent 1,2-dioxetane
compound as recited in claim 1.

32. The process of claim 31 in which the process carried out is a step in an immunoassay.

33. The process of claim 32 in which the immunoassay is for the detection of a specific binding pair comprising an antigen and an antibody.

34. The process of claim 32 in which the label used in the assay is an enzyme.

35. The process of claim 32 in which the label used in the assay is the chemiluminescent 1,2-dioxetane compound.

36. The process of claim 32 in which the chemiluminescent 1,2-dioxetane compound is enzymatically cleavable.

37. The process of claim 32 in which the chemiluminescent 1,2-dioxetane compound is chemically cleavable.

38. The process of claim 32 in which the immunoassay is for the detection of an enzyme.

39. The process of claim 32 in which the immunoassay is for the detection of a hormone.

40. The process of claim 31 in which the process carried out is a step in a chemical assay.

41. The process of claim 40 in which the chemical assay is for the detection of a chemical substance which, during the assay, is caused to decompose to form a substance capable of causing the chemiluminescent 1,2-dioxetane compound to decompose.

42. The process of claim 41 in which the chemical substance is glucose.

43. The process of claim 41 in which the chemical substance is cholesterol.

44. The process of claim 31 in which the process carried

out is a nucleic acid probe assay.

45. The process of claim 44 in which the nucleic acid probe assay is for the detection of a virus.

46. The process of claim 31 in which the process carried out is a histocompatibility assay.

47. The process of claim 31 in which the process carried out is a technique for studying the microstructure of a macromolecule.

48. The process of claim 31 in which the process carried out is a multi-channel assay carried out in the presence of at least two of the chemiluminescent 1,2-dioxetane compounds as recited in claim 1 as substrates, each of which upon
5   decomposition emits light of a different wavelength from the other(s) and each of which has a labile ring substituent cleavable by a different means from the other(s).

49. The process of claim 31 in which the process carried out is a multi-channel assay carried out in the presence of at least one of the chemiluminescent 1,2-dioxetane compounds as recited in claim 1 and at least one other chemiluminescent
5   compound as substrates, each of which chemiluminescent 1,2-dioxetane compounds and other chemiluminescent compounds emit light of a different wavelength from the other(s) and each of which is cleavable by different means from the other(s) to produce a light-emitting fluorophore moiety.

50. The process of claim 49 in which the multi-channel assay is an immunoassay for the quantitation of human chorionic gonadotropin and human luteinizing hormone contained in a single sample and is carried out in the presence of 3-(2'-

5   spiroadamantane)-4-methoxy-4-(3'-phosphoryloxy)phenyl-1,2-dioxetane disodium salt and 3-(2'-spiroadamantane)-4-methoxy-4-(7"-acetoxy)naphth-2'-yl-1,2-dioxetane as the chemiluminescent substrates.

51. The process of claim 50 in which the phosphoryl-oxyphenyl dioxetane chemiluminescent substrate is cleaved by alkaline phosphatase conjugated to mouse monoclonal anti-β-HCG and the acetoxynaphthyl dioxetane chemiluminescent substrate is

5   cleaved by carboxylesterase conjugated to mouse monoclonal anti-HLH.

52. The process of claim 49 in which the multi-channel assay is a nucleic acid probe assay for the quantitation of herpes simplex virus, cytomegalovirus and human papiloma virus contained in a single sample and is carried out in the presence

5   of 3-(2'-spiroadamantane)-4-methoxy-4-(3'-phosphoryloxy)phenyl-1,2-dioxetane disodium salt, 3-(2'-spiroadamantane)-4-methoxy-4-(7"-acetoxy)naphth-2'-yl-1,2-dioxetane and 3-(2'-spiroadamantane)-4-methoxy-4-(5"-β-galactosyloxy)naphth-2'-yl-1,2-dioxetane as the chemiluminescent substrates.

53. The process of claim 52 in which the phosphoryloxyphenyl dioxetane chemiluminescent substrate is cleaved by a DNA probe for HSV labeled with alkaline phosphatase, the acetoxynaphthyl dioxetane chemiluminescent substrate is

5   cleaved by a DNA probe for CMV labeled with carboxylesterase and the β-galactosyloxynaphthyl dioxetane chemiluminescent substrate is cleaved by a DNA probe for HPV labeled with β-galactosidase.

54. A fused polycyclic hydrocarbon ring-containing fluorophoric compound having from 9 to 30 carbon atoms,

inclusive, and having a halo substituent and an ether substituent

or a hydroxy substituent, the total number of ring carbon atoms

5   separating said two substituents, including the carbon atoms to

which said substituents are attached, being an odd whole number.

55.   A compound as recited in claim 54 which is a fused

polycyclic aromatic hydrocarbon ring-containing fluorophoric

compound in which said halo substituent is an iodine atom and

said ether substituent is an alkoxy group.

56.   2-Iodo-7-methoxynaphthalene.

57.   1-Iodo-8-methoxynaphthalene.

58.   2-Iodo-5-methoxynaphthalene.

59.   1-Iodo-6-methoxynaphthalene.

60.   2-Iodo-10-methoxyanthracene.

61.   A compound as recited in claim 54 having a hydroxy

substituent and in which said halo substituent is an iodine atom.

62.   2-Iodo-7-hydroxynaphthalene.

63.   1-Iodo-8-hydroxynaphthalene.

64.   2-Iodo-5-hydroxynaphthalene.

65.   1-Iodo-6-hydroxynaphthalene.

66.   2-Iodo-10-hydroxyanthracene.

67.   A compound having the general formula:

$$T-\underset{H}{\overset{OH}{\underset{|}{C}}}-R_4$$

wherein T is an unsubstituted or substituted cycloalkyl, aryl,

heteroaryl, alkoxy, aryloxy, alkoxyalkoxy, cycloalkylidene or

fused polycycloalkylidene group and R_4 is the residue of a fused polycyclic hydrocarbon ring-containing fluorophoric compound having from 9 to about 30 carbon atoms, inclusive, and having an ether substituent, the total number of carbon atoms separating the ring carbon atom to which said ether substituent is attached and the ring carbon atom through which R_4 is attached to the remainder of the compound, including the carbon atoms at said point of attachment, being an odd whole number.

68. A compound as recited in claim 67 in which R_4 is the residue of a fused polycyclic aromatic hydrocarbon ring-containing fluorophoric compound in which said ether substituent is an alkoxy group.

69. 7-Methoxynaphth-2-yladamant-2'-yl alcohol.

70. 8-Methoxynaphth-2-yladamant-1'-yl alcohol.

71. 5-Methoxynaphth-2-yladamant-2'-yl alcohol.

72. 6-Methoxynaphth-2-yladamant-1'-yl alcohol.

73. A compound having the general formula:
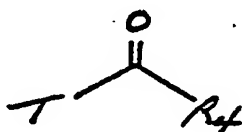
wherein T is an unsubstituted or substituted cycloalkyl, aryl, heteroaryl, alkoxy, aryloxy, alkoxyalkoxy, cycloalkylidene or fused polycycloalkylidene group and R_4 is the residue of a fused polycyclic hydrocarbon ring-containing fluorophoric compound having from 9 to about 30 carbon atoms, inclusive, and having an ether substituent, the total number of carbon atoms separating the ring carbon atom to which said ether substituent is attached

10 and the ring carbon atom through which R_4 is attached to the remainder of the compound, including the carbon atoms at said points of attachment, being an odd whole number.

74. A compound as recited in claim 73 in which R_4 is the residue of a fused polycyclic aromatic hydrocarbon ring-containing fluorophoric compound in which said ether substituent is an alkoxy group.

75. 7-Methoxynaphth-2-yladamant-2'-yl ketone.

76. 8-Methoxynaphth-2-yladamant-1'-yl ketone.

77. 5-Methoxynaphth-2-yladamant-2'-yl ketone.

78. 6-Methoxynaphth-2-yladamant-1'-yl ketone.
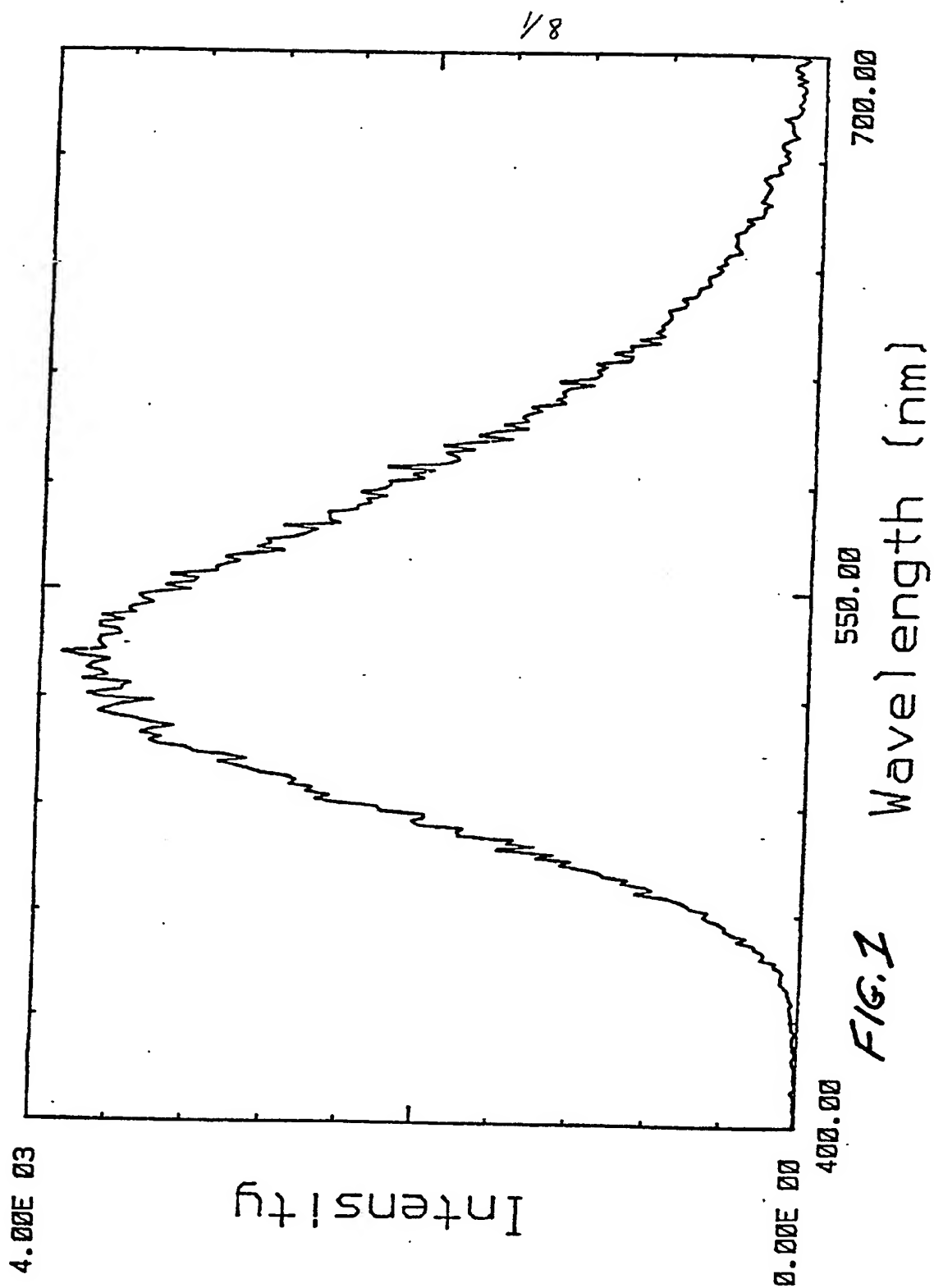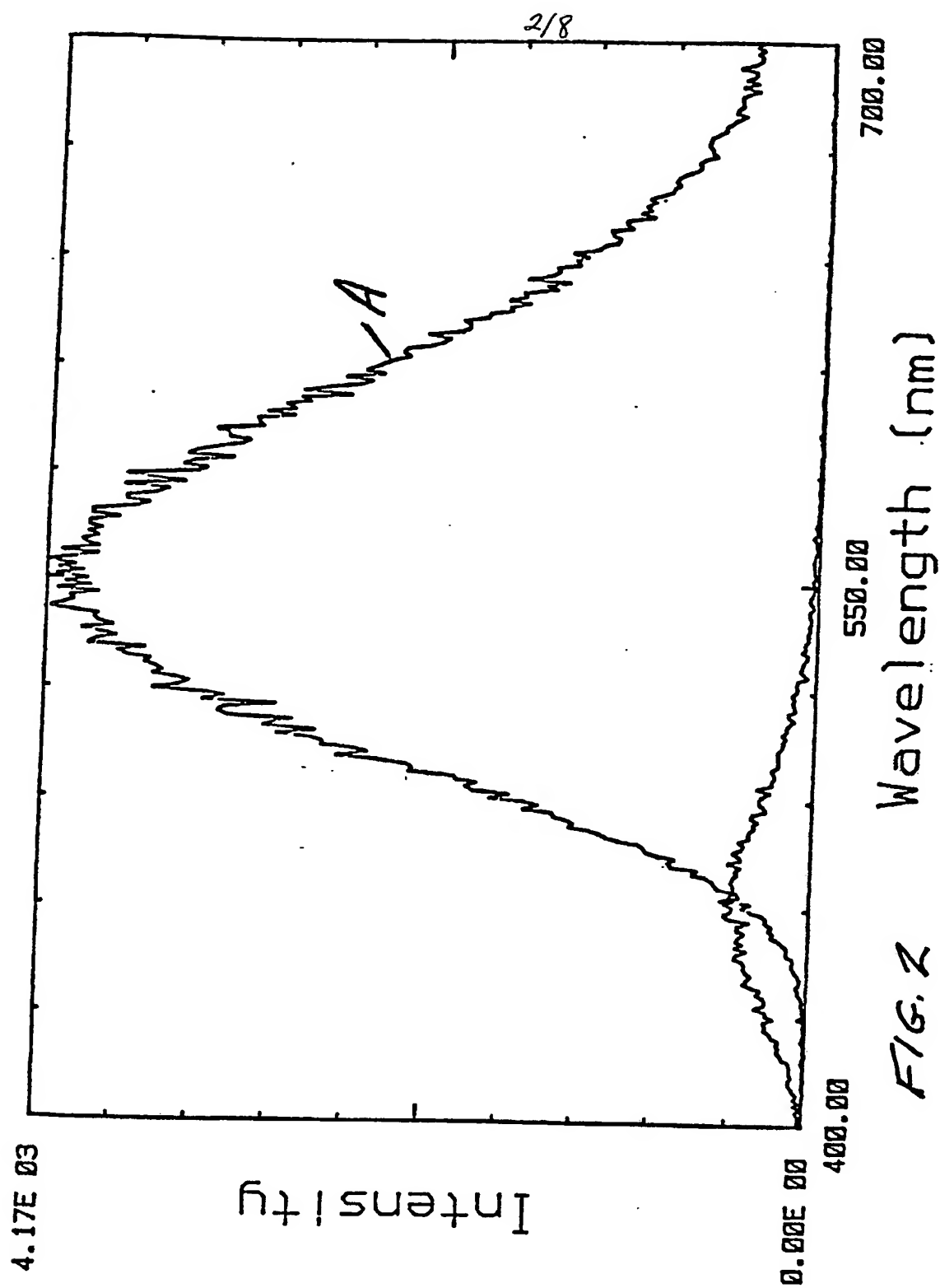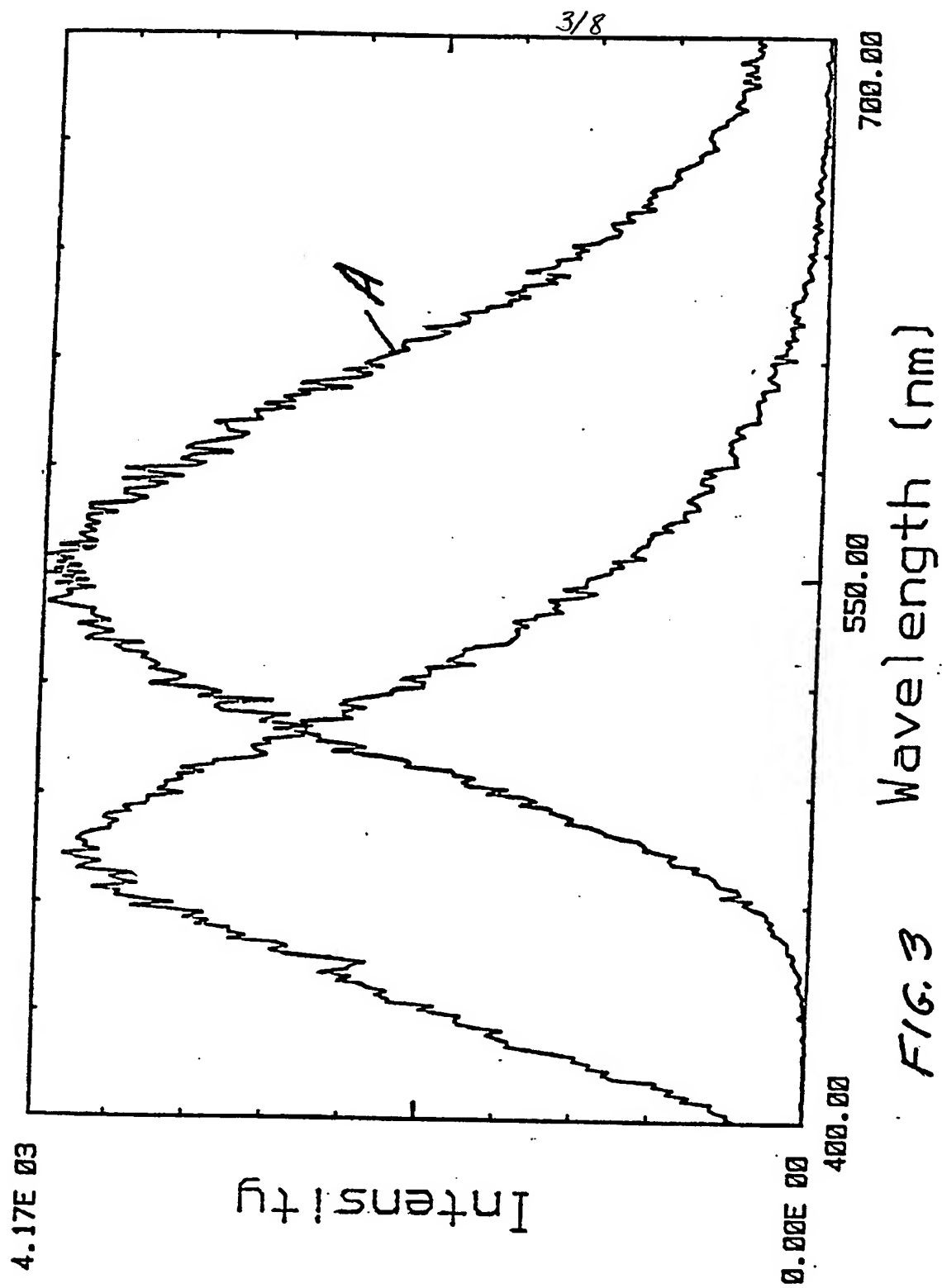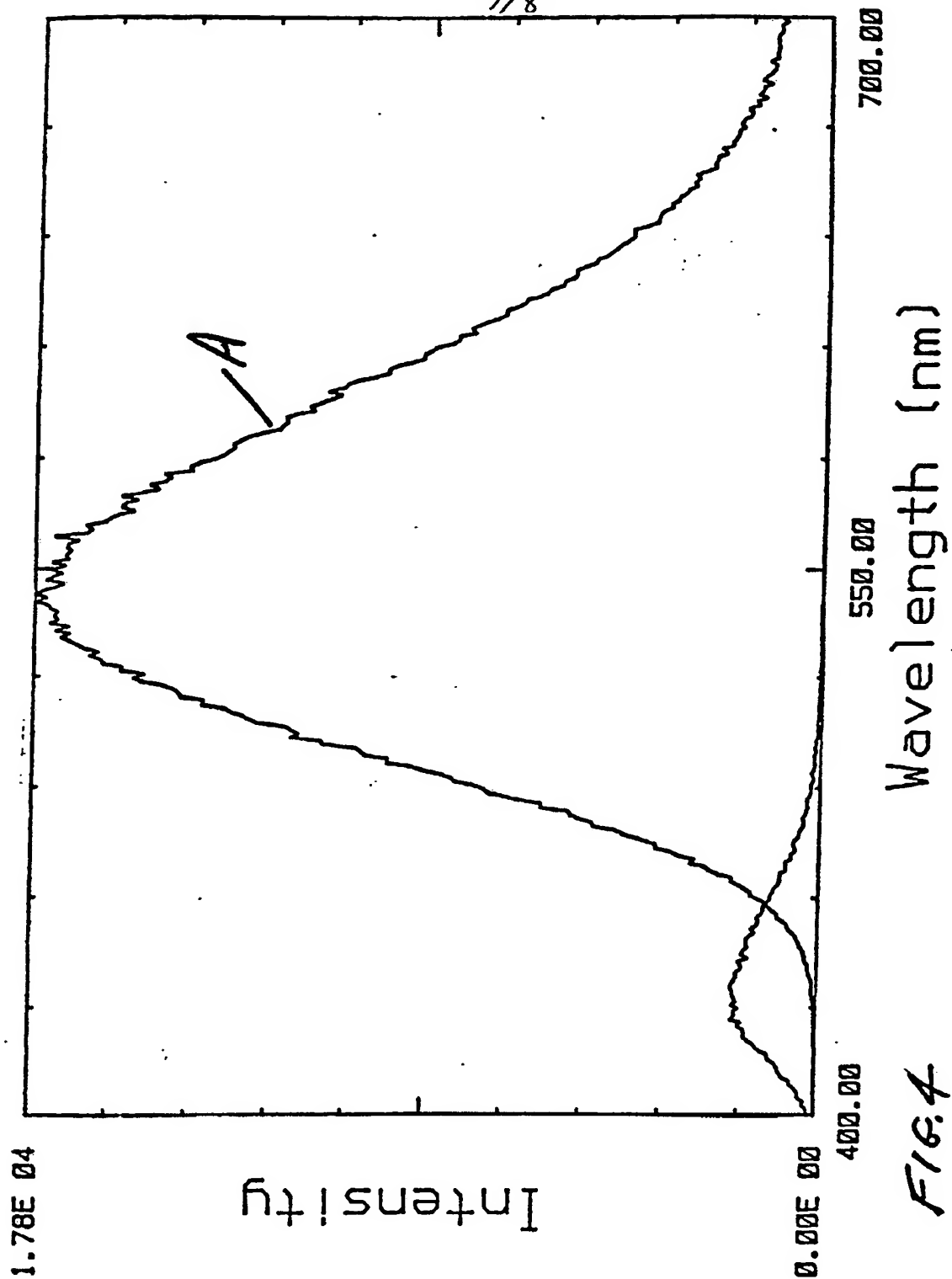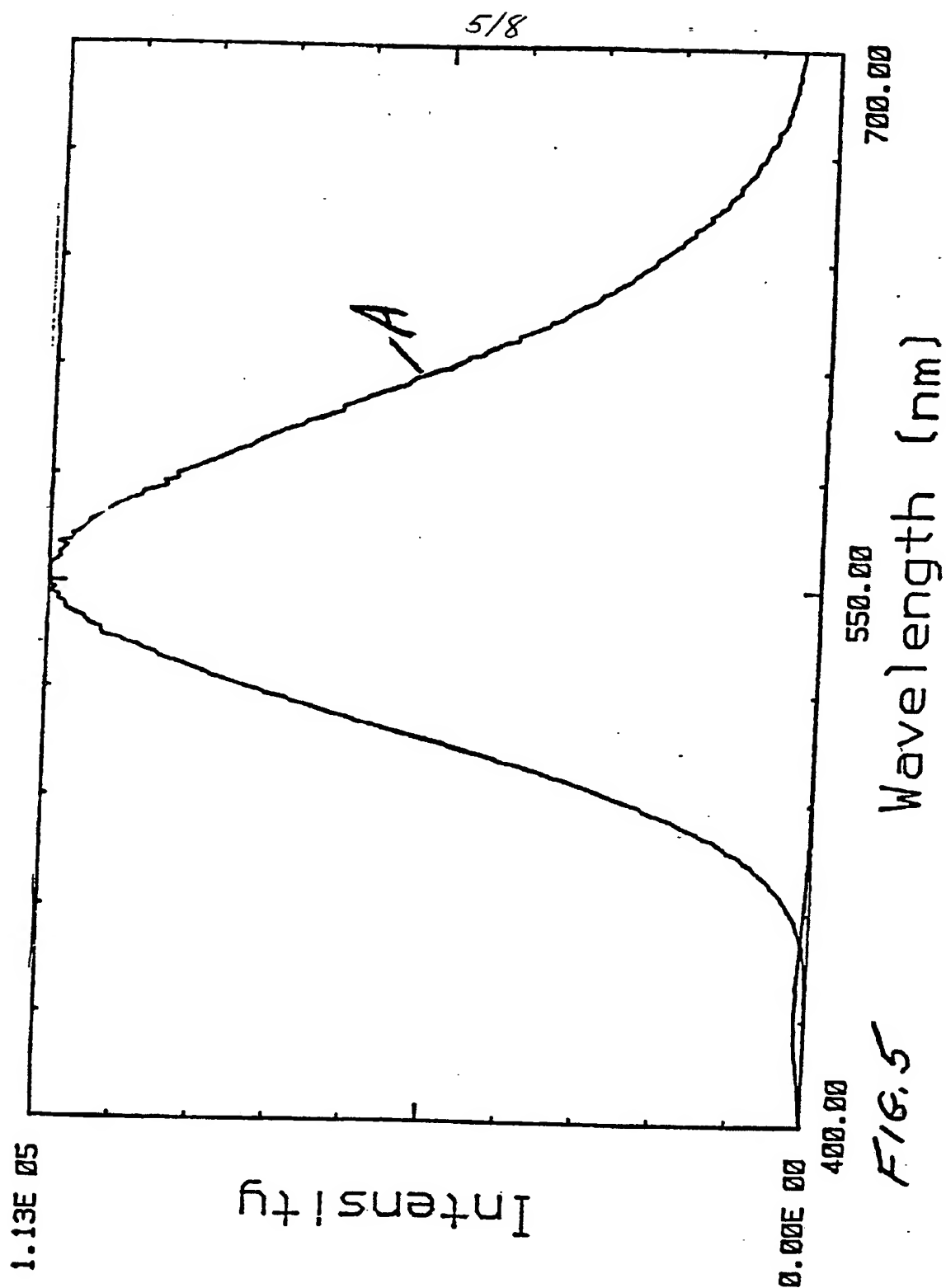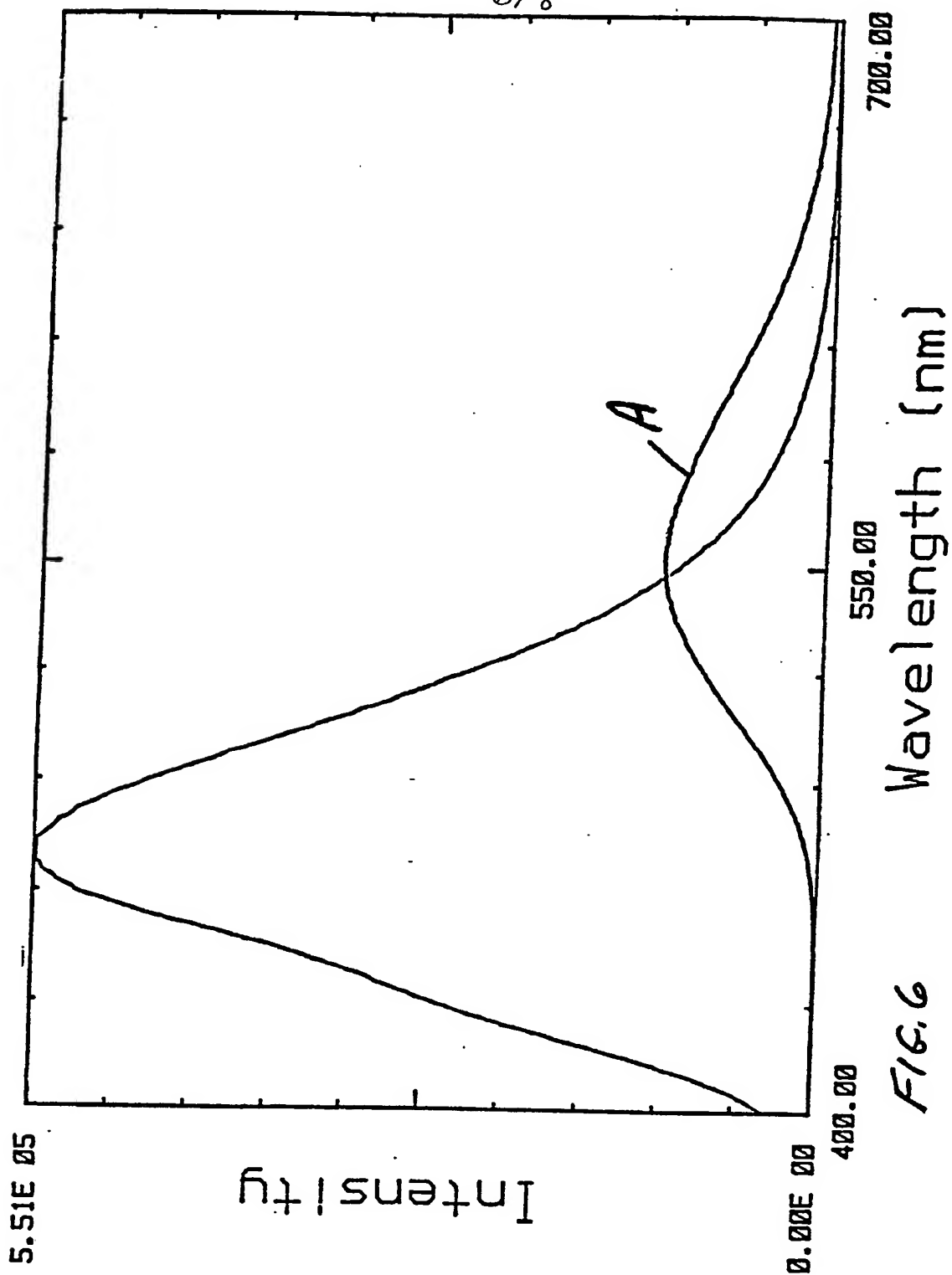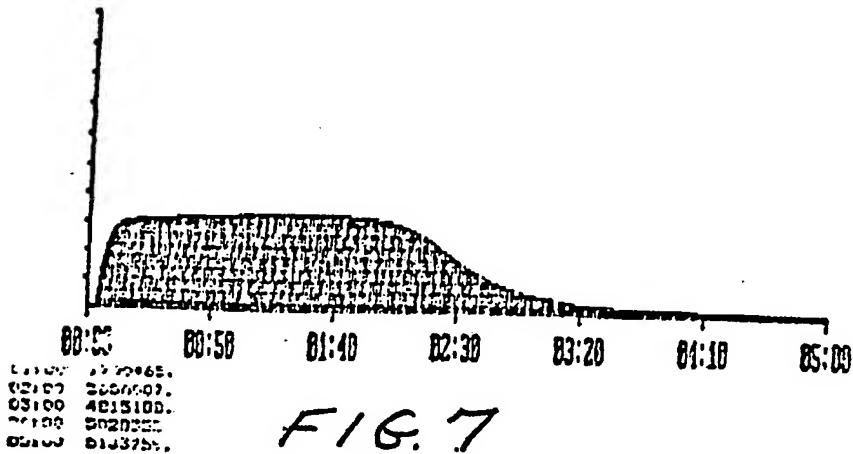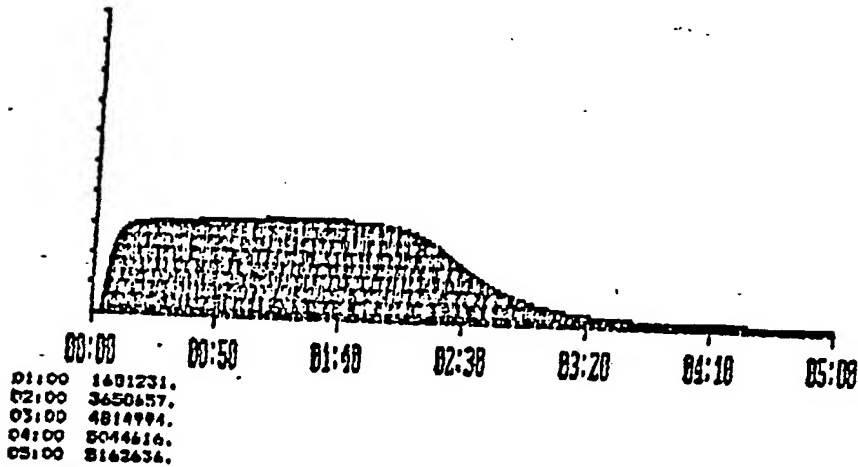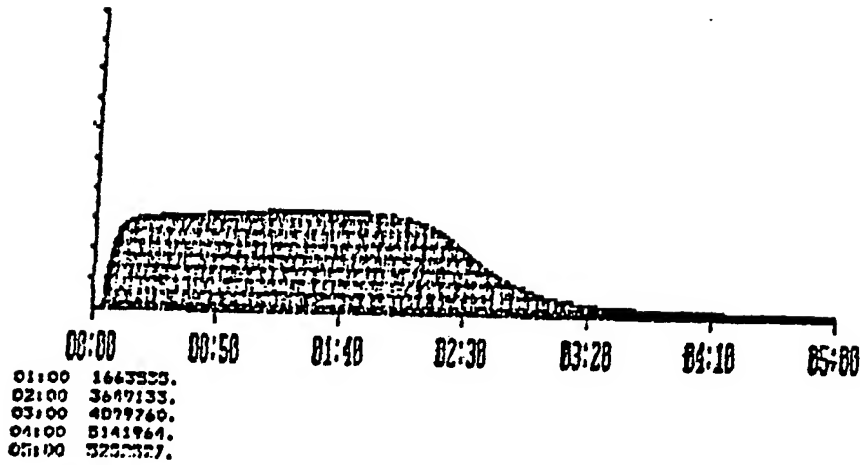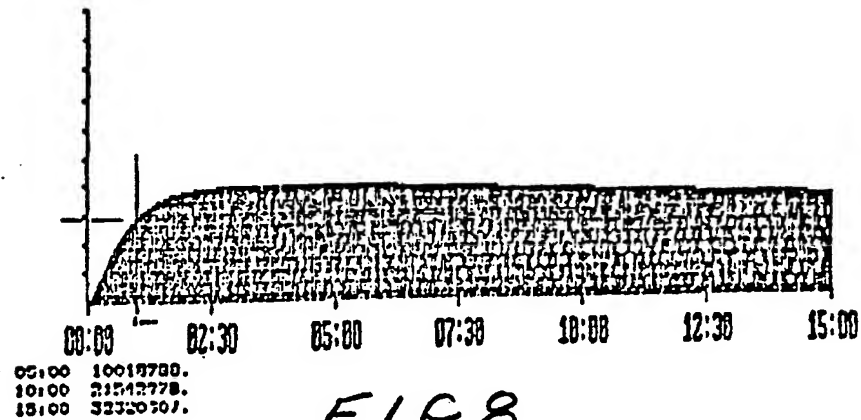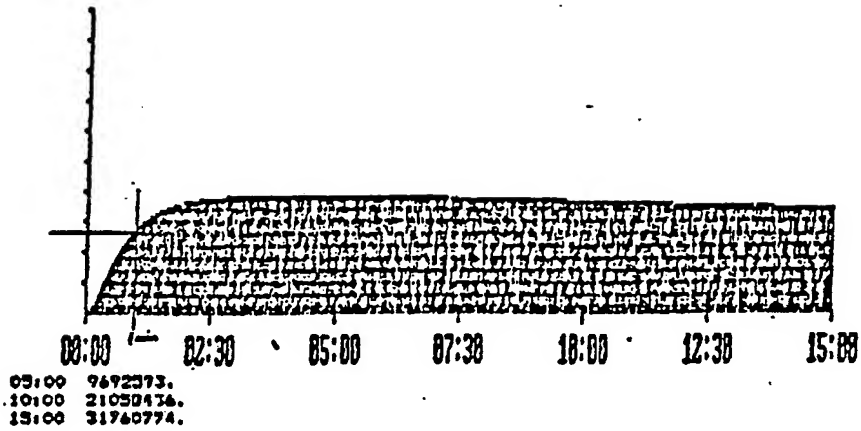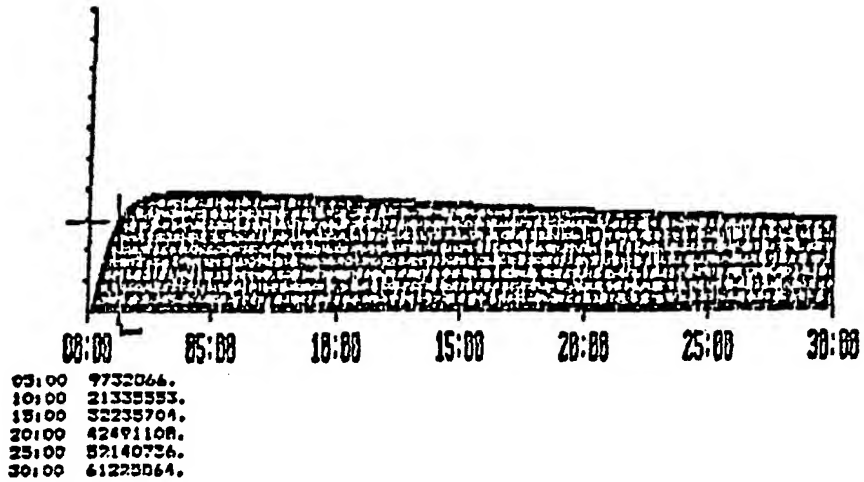
1/8

FIG.1

FIG. 2

FIG. 3

FIG.4

FIG.5

FIG.6

01:00 1663323.
02:00 3649135.
03:00 4079760.
04:00 5141764.
05:00 5299527.

01:00 1601231.
02:00 3650657.
03:00 4814994.
04:00 5044616.
05:00 5162634.

01:00 1720965.
02:00 3620607.
03:00 4815100.
04:00 5020000.
05:00 5133755.

FIG. 7

05:00	9752066.
10:00	21335553.
15:00	32235704.
20:00	42491108.
25:00	52140736.
30:00	61225064.

05:00	9692373.
10:00	21050436.
15:00	31760774.

05:00	10018700.
10:00	21542778.
15:00	32320507.

FIG. 8

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): C07C 39/14; 43/225; 43/23; 49/788; C07D 321/00; See Continuation sheet 2
U.S.Cl.: 435/4,5,7; 436/172,519,537; 546/152; See Continuation sheet 2

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
U.S.Cl.:	435/4,5,7; 436/172,519,537; 546/152; 549/214,221,510,511; 568/328,632,634,737

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched 8

CAS ONLINE: GRAPHIC STRUCTURES

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
X	EP, A, WO 88/0254051 (SCHAAP ET AL) 27 January 1988. See Abstract.	1-53
X	Tetrahedron Letters, Vol. 28 No. 11, Schaap et al (1987) "Chemical and Enzymatic Triggering of 1,2-Dioxetanes" pages 1155-1158 See page 1155.	1-53
X	Chemical Abstracts Vol. 81, No. 9, issued 02 September 1974, Columbus. Ohio, USA Hellwinkel et al, Stabilization of the trigonal bipyramidal ground states of spirocyclic pentaorganylphosphoranes and -arsoranes, abstract No. 49097y.	54-60
X	Chemical Abstracts, Vol. 87 No. 13, issued 26 September 1977, Columbus, Ohio, USA Parra et al. Synthesis of azasteroids, Abstract No. 102512g.	54-60
A	JP, B, 7306526 TOA GOSEI CHEMICAL 04 June 1970 See Abstract.	54,61-66

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
15 SEPTEMBER 1989	07 NOV 1989

International Searching Authority	Signature of Authorized Officer
ISA/US	RICHARD L. RAYMOND

Form PCT/ISA/210 (second sheet) (Rev.11-87)

CGK00011589

PCT/US89/02793
I. CLASSIFICATION OF SUBJECT MATTER (CONTINUED)
CONTINUATION SHEET 2

IPC(4): C07F 7/18, 9/12; C12Q 1/00, 1/70; G01N 21/76, 33/542,
33/554, 53/00

U.S.Cl.: 549/214, 221, 510, 511; 568/328, 632, 634, 737

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V.☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1.☐ Claim numbers _____ , because they relate to subject matter [12] not required to be searched by this Authority, namely:

2.☐ Claim numbers _____ , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3.☐ Claim numbers _____ , because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI.☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

Telephone Practice

   I   Claims 1-53 drawn to products and process of using to generate light; class 435 subclass 7.

               (See Continuation sheet 1)

1.☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2.☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3.☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4.☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

II.   Claims 54-66 drawn to naphthalene intermediates;
class 568 subclass 634.

III.   Claims 67-72 drawn to alcohol intermediates; class
568 subclass 632.

IV.   Claims 73-78 drawn to ketone intermediates; class
568 subclass 328.